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<b>(54) Title:</b> CROSSLINKED PROTEIN CRYSTALS AS UNIVERSAL SEPARATION MEDIA		
<b>(57) Abstract</b> <p>The present invention relates to the use of crosslinked protein crystals in methods, apparatus and systems for separating a substance or molecule of interest from a sample. According to a preferred embodiment of this invention, crosslinked protein crystals are used in chromatographic methods, apparatus and systems in which separation is based on a physical or chemical property of that substance or molecule of interest. Advantageously, the crosslinked protein crystals which characterize the methods, apparatus and systems of this invention possess excellent mechanical strength and well developed porous structure, demonstrate significant affinity and chiral selectivity and are extremely stable in aqueous and organic solvents. These properties render the crystals particularly useful as sorbents for separations, including size exclusion, affinity and chiral chromatography.</p>		

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CROSSLINKED PROTEIN CRYSTALS  
AS UNIVERSAL SEPARATION MEDIA

TECHNICAL FIELD OF THE INVENTION

The present invention relates to the use of  
5 crosslinked protein crystals in methods, apparatus and  
systems for separating a substance or molecule of  
interest from a sample. According to a preferred  
embodiment of this invention, crosslinked protein  
crystals are used in chromatographic methods, apparatus  
10 and systems in which separation is based on a physical  
or chemical property of that substance or molecule of  
interest.

Advantageously, the crosslinked protein  
crystals which characterize the methods, apparatus and  
15 systems of this invention possess excellent mechanical  
strength and well developed porous structure,  
demonstrate significant affinity and chiral selectivity  
and are extremely stable in organic solvents and  
aqueous-organic solvent mixtures. These properties  
20 render the crystals particularly useful as sorbents for  
separations, including size exclusion, affinity and  
chiral chromatography.

- 2 -

### BACKGROUND OF THE INVENTION

One of the most important scientific achievements of the 20th century was the discovery and development of chromatography for the analysis, separation and purification of different low [M.S. Tswett, "Berichte der deutschen botanischen Gesellschaft", Bd. 24, S. 316-23 (1906)] and high [C. Claesson, "Discussion of the Faraday Society", N 7, (1949)] molecular weight substances -- from optical isomers to transuranium isotopes and polymer macromolecules. Both analytical and preparative scale chromatographic techniques are widely used in the biosciences, chemistry, polymer science, nuclear physics, as well as in the petroleum, polymer, pharmaceutical, agrochemical, biotechnology and food industries.

Chromatography is a variation of a dynamic adsorption process in a two-phase system, in which a mixture of substances or a crude substance migrates through a porous medium with a gas or solvent flow. Individual components of the mixture or, contaminants from the crude substance, are separated according to their respective sorption activities [B.G. Belenkii and L.Z. Vilenchik, Modern Liquid Chromatography of Macromolecules, Elsevier Press, Amsterdam-Oxford-New York-Tokyo (1983); J.C. Giddings, Dynamics of Chromatography, Marcel Dekker, New York (1965); L.R. Snyder and J.J. Kirkland, Introduction to Modern Liquid Chromatography, Wiley-Interscience, New York (1979)]. Depending on the type of mobile phase used,

- 3 -

chromatography is divided into gas and liquid chromatography. It is also divided according to the type of sorbent used as the stationary phase. Such chromatography types include size exclusion [J. Porath and P. Flodin, Nature, 183, p. 1651 (1959); J.C. Moore, J. Polym. Sci., A, p. 835 (1964)], affinity [J.A. Jonsson, Ed., Chromatographic Theory and Basic Principles, Marcel Dekker, New York (1987)] and chiral chromatography [J. Hermansson et al., in M. Ziefand and L. Crane, Eds., Chromatographic Chiral Separations, 40, pp. 245-81, Marcel Dekker, New York (1987)].

Macroporous sorbents, which are adsorptionally inert with respect to the molecules of the substances undergoing chromatography, are used in size exclusion chromatography to separate molecules according to their hydrodynamic size. Usually, the method is applied to macromolecules ranging from oligomers (with molecular weights of a few 100 to 10,000 daltons) to polymers (with molecular weights of 10,000 to a few million daltons). Several types of stationary phases are employed in size exclusion chromatography. Macromolecular crosslinked swelling polymer gels, macroporous polymer sorbents and macroporous silicate sorbents are those primarily used [Belenkii and Vilenchik, supra].

Ion exchange chromatography separates molecules, such as proteins, that have an electrical charge. This type of chromatography is widely used for preparative scale separations.

Different types of adsorption chromatography are employed to separate and analyze low molecular weight substances. Adsorption chromatography is

- 4 -

divided into three major categories, according to the interaction between the stationary phase and molecules of the substances to be separated: reverse-phase chromatography (hydrophobic interaction with bonded  
5 non-polar groups of the stationary phase); affinity chromatography (biospecific sorption) and chiral chromatography (steric interactions that can involve hydrogen bonding, dipole-dipole,  $\pi$ - $\pi$ , electrostatic and hydrophobic interaction). Affinity chromatography  
10 sorbents are typically porous materials [P. Mohr, Affinity Chromatography, Marcel Dekker, New York (1985)]. Among chiral separation phases, sorbents with immobilized proteins are among the most important [M.J. Gattuso et al., Proceedings of the Chiral 95 USA,  
15 Springs Innovations, Ltd. Boston, pp. 51-55 (1995)].

Chiral chromatography is of special interest to the industrial-scale synthesis of specialty chemicals, pharmaceuticals and agrochemicals. Many drugs and pesticides contain at least one chiral  
20 center. Enantiomeric purity is an important factor in the biological activity and safety of these materials. The FDA has called for complete pharmacodynamics and pharmacokinetics on the individual isomers of proposed pharmaceutical agents. As a result, most new chiral  
25 drugs will be developed as a single enantiomer.

According to recent projections, the portion of synthetic chiral pharmaceuticals introduced as single enantiomers will continue to increase during this decade and may reach 80% by the year 2000 [R.L.  
30 Bratzler and J.W. Young, "Enzymes and their Relative Importance in Chiral Chemistry", in Opportunities with Industrial Enzymes, R. Heinemann and B. Wolnak, Eds.,

- 5 -

Johnson Graphics, Decatur, Illinois, pp. 8-20, (1992); J.H.G.M. Mustaers and H.J. Kooreman, Rec. Trav. Chim. Pays-Bas, 110, pp. 185-88 (1991)]. The focus of the FDA in approving single drug isomers is expected to

5 result in new drugs being developed as single isomers and many existing drugs being converted to single isomers. Effective methods for the manufacture and analysis of homochiral materials is important to the development of single isomer drugs and agrochemicals.

10 The chiral sector of the pharmaceutical industry currently constitutes about \$15 billion in sales and is expected to reach \$150 billion by the end of the century [Bratzler and Young, supra]. Chiral chromatography and chiral stationary phases will play

15 an important role in producing and analyzing many of the fine chemicals or intermediates used as optically pure drugs, especially early on in the drug development cycle.

While existing sorbents can separate a wide

20 variety of compounds according to their molecular weight, adsorption, affinity or chiral properties, there are several limitations that generally preclude wider applications of chromatography. These limitations include low column loading, especially for

25 protein-based chiral stationary phases. Typically, the fact that the chiral recognition protein is chemically bound to a silica support means that the bulk of the column volume is occupied by the support, not by the chiral selector. Low column loading makes chiral

30 stationary phases expensive for preparative separations.

- 6 -

A second limitation relates to eluents. The majority of current chiral stationary phases is designed for normal phase chromatography and utilizes mixtures of water miscible organic solvents, such as  
5 hexane/2-propanol. These mixtures are incompatible with eluents for reverse phase chromatography, such as acetonitrile or methanol/water, making it difficult to use both types of chromatography columns in the same instrument.

10 A third limitation is narrow operational conditions. Protein chiral stationary phases are effective only in a narrow range of conditions. For example, an Ultron ES-OVM column can function in the range of pH 3-7.5 (the same limitation exists for  
15 Pirkle phases), at temperatures lower than 40°C and in eluents containing less than 50% organic solvent.

A fourth limitation relates to high cost. The price of the majority of small analytical chiral chromatography columns is between \$1,000 - \$2,000. The  
20 price of bulk chiral stationary phase is around \$8,000 per kg, making it the greatest single element of the cost of chiral chromatographic separations.

PCT patent application WO95/09907 relates to methods using crystals of biological macromolecules to  
25 separate compounds from mixtures, based on selective binding affinity. To date, however, the chromatography features of protein crystals have not been commercially exploited, due to widely held misconceptions. These include perceived difficulties of protein crystal  
30 preparation on large scales; perceived general instability of protein crystals and perceived mechanical fragility of protein crystals [M.A. Navia



- 7 -

et al., Stability and Stabilization of Enzymes, W.J.J. van den Tweel et al. Eds., Elsevier, Amsterdam, pp. 63-73 (1993)].

The advent of crosslinked enzyme crystal  
5 ("CLEC®") technology put an end to misconceptions surrounding protein crystal preparation [N.L. St. Clair and M.A. Navia, "Cross-Linked Enzyme Crystals as Robust Biocatalysts", J. Am. Chem. Soc., 114, pp. 7314-16 (1992)]. Crosslinked enzyme crystals retain their  
10 activity in environments that are normally incompatible with enzyme (soluble or immobilized) function. Such environments include prolonged exposure to high temperature and extreme pH. Additionally, in organic solvents and aqueous-organic solvent mixtures,  
15 crosslinked enzyme crystals exhibit both stability and activity far beyond that of their soluble or conventionally-immobilized counterparts. Crosslinked enzyme crystals represent an important advance in the area of biocatalysis, as attractive and broadly  
20 applicable catalysts for organic synthesis reactions [R.A. Persichetti et al., "Cross-Linked Enzyme Crystals (CLECs) of Thermolysin in the Synthesis of Peptides", J. Am. Chem. Soc., 117, pp. 2732-37 (1995) and J.J. Lalonde et al., "Cross-Linked Crystals of Candida  
25 rugosa Lipase: Highly Efficient Catalysts for the Resolution of Chiral Esters", J. Am. Chem. Soc., 117, pp. 6845-52 (1995)]. CLEC® technology represents a general stabilization technology, providing proteins characterized by increased stability against heat,  
30 organic solvents and exogenous proteolysis [C. Govardhan and A.L. Margolin, "Extremozymes for Industry

- 8 -

- from Nature and by Design", Chemistry and Industry, pp. 689-93 (1995)].

Currently, five crosslinked enzyme crystal modifications -- PeptiCLEC™-TR for racemization free peptide synthesis and ChiroCLEC™-CR [J.J. Lalonde et al., J. Am. Chem. Soc., 117, pp. 6845-52 (1995)], ChiroCLEC™-PC and ChiroCLEC™-BL for resolution of esters, acids, amines and alcohols and ChiroCLEC™-EC for resolution of amines, amino acids and esters and regioselective modification -- are routinely produced on a kilogram scale and are commercially available in different crystal forms [A.L. Margolin, "Novel Crystalline Catalysts", Trends. Biotech., 14, pp. 223-30 (1996)].

Aside from their use as reagents in chemical and biochemical processes, such crosslinked protein crystals also represent a means for satisfying the present need for commercially and technically feasible separation media for techniques, devices and systems, including those based on chromatography.

#### DISCLOSURE OF THE INVENTION

The present invention provides methods, apparatus and systems using crosslinked protein crystals for separating a substance or molecule of interest from a sample, based on a physical or chemical property of the substance or molecule of interest. Such separation methods are characterized by the step of contacting the crosslinked protein crystals with the substance or molecule of interest by any means, for a sufficient time and under conditions which permit said

- 9 -

protein to retain said substance or molecule of interest, thus separating it from said sample.

According to this invention, crosslinked protein crystals may be used in place of or in  
5 combination with conventional sorbents in methods, devices and systems for separating molecules of interest from mixtures or crude preparations thereof. Separation methods, apparatus and systems using crosslinked protein crystals according to this  
10 invention are advantageously characterized by higher degrees of sensitivity, volumetric productivity and throughput than those of methods, apparatus and systems based on conventional sorbents.

Chromatographic techniques and systems which  
15 may be carried out using crosslinked protein crystals include size exclusion chromatography, adsorption chromatography (for example, reverse phase chromatography), affinity chromatography and chiral chromatography. Such types of chromatography may be  
20 advantageously carried out in the presence of an aqueous solvent, organic solvent or an aqueous-organic solvent mixture.

#### BRIEF DESCRIPTION OF THE DRAWINGS

25 Figure 1 is a graph representing the pore size distribution of crosslinked thermolysin crystals (TR-3) useful in this invention.

Figure 2 is a graph representing the distribution coefficient as a function of the root mean  
30 square radius of polyethylene glycol ("PEG")

- 10 -

macromolecules run on a chromatographic column packed with crosslinked thermolysin crystals (TR-3).

Figure 3 is a graph representing the relationship between the root mean square radius of PEG  
5 macromolecules and retention times on a chromatographic column packed with crosslinked thermolysin crystals (TR-3).

Figure 4 is a graph representing the relationship between the molecular weight of PEG  
10 macromolecules and retention times on a chromatographic column packed with crosslinked thermolysin crystals (TR-3).

Figures 5 and 6 are chromatograms illustrating the separation of PEG samples by size  
15 exclusion chromatography on a chromatographic column packed with crosslinked thermolysin crystals (TR-3). Figure 5 is a chromatogram of three PEG standards with average molecular weights of 62, 600 and 10,000 daltons. Figure 6 illustrates the separation of six  
20 PEG standards with average molecular weights of 62, 106, 194, 400, 1,500 and 10,000 daltons in 50% acetonitrile.

Figure 7 is a chromatogram illustrating the separation of phenylacetic acid, hydroxyphenylglycine  
25 and R-phenylglycine by affinity chromatography on a chromatographic column packed with crosslinked thermolysin crystals (TR-3).

Figure 8 is a chromatogram illustrating the separation of phenylacetic acid, hydroxyphenylglycine,  
30 R-phenylglycine and L-phenylalaninamide by affinity chromatography on a chromatographic column packed with crosslinked thermolysin crystals (TR-3).

- 11 -

Figure 9 is a chromatogram illustrating the separation of a mixture of D-isomer of phenyllactic acid and ibuprofen by affinity chromatography on a chromatographic column packed with crosslinked thermolysin crystals (TR-3).

Figure 10 is a chromatogram of flurbiprofen on a chromatographic column packed with crosslinked thermolysin crystals (TR-3).

Figure 11 is a chromatogram illustrating the separation of a mixture of flurbiprofen and ibuprofen by affinity chromatography on a chromatographic column packed with crosslinked thermolysin crystals (TR-3).

Figure 12 is a chromatogram illustrating the separation of a mixture of the D and L optical isomers of phenyllactic acid by chiral chromatography on chromatographic columns packed with crosslinked thermolysin crystals (TR-2) and (TR-3) in series.

Figure 13 is a chromatogram illustrating the separation of a mixture of the D and L optical isomers of phenyllactic acid by chiral chromatography on a chromatographic column packed with crosslinked thermolysin crystals (TR-2).

Figure 14 is a chromatogram illustrating the separation of a mixture of the R and S optical isomers of phenylglycine by chiral chromatography on chromatographic columns packed with crosslinked thermolysin crystals (TR-2) and (TR-3) (injection volume 10  $\mu$ l).

Figure 15 is a chromatogram illustrating the separation of a mixture of the R and S optical isomers of phenylglycine by chiral chromatography on chromatographic columns packed with

- 12 -

crosslinked thermolysin crystals (TR-2) and (TR-3) (injection volume 1.5  $\mu$ l).

Figure 16 is a graph representing the pore size distribution of crosslinked Candida rugosa lipase crystals useful in this invention.

Figure 17 is a graph representing the distribution coefficient as a function of the root mean square radius of PEG macromolecules run on a chromatographic column packed with crosslinked Candida rugosa lipase crystals.

Figure 18 is a graph representing the relationship between the root mean square radius of PEG macromolecules and retention times on a chromatographic column packed with crosslinked Candida rugosa lipase crystals.

Figure 19 is a graph representing the relationship between the molecular weight of PEG macromolecules and retention times on a chromatographic column packed with crosslinked Candida rugosa lipase crystals.

Figure 20 is a chromatogram illustrating the separation of PEG samples by size exclusion chromatography on a chromatographic column packed with crosslinked Candida rugosa lipase crystals. The figure includes chromatograms of four PEG standards with average molecular weights of 62, 400, 1,500 and 10,000 daltons.

Figure 21 is a chromatogram illustrating the separation of PEG samples by size exclusion chromatography on a chromatographic column packed with crosslinked Candida rugosa lipase crystals. The figure includes chromatograms of six PEG standards with

- 13 -

average molecular weights of 62, 106, 194, 400, 1,500 and 10,000 daltons separated in 50% acetonitrile.

Figure 22 is a chromatogram illustrating the separation of PEG samples by size exclusion

5 chromatography on a chromatographic column packed with crosslinked Candida rugosa lipase crystals. The figure includes chromatograms of four PEG standards with average molecular weights of 194, 900, 1,500 and 5,000 daltons.

10 Figure 23 is a graph representing the pore size distribution of crosslinked Pseudomonas cepacia lipase crystals useful in this invention.

Figure 24 is a graph representing the distribution coefficient as a function of the root mean square radius of PEG macromolecules run on a chromatographic column packed with crosslinked Pseudomonas cepacia lipase crystals.

Figure 25 is a graph representing the relationship between the root mean square radius of PEG macromolecules and retention times on a chromatographic column packed with crosslinked Pseudomonas cepacia lipase crystals.

Figure 26 is a graph representing the relationship between the molecular weight of PEG macromolecules and retention times on a chromatographic column packed with crosslinked Pseudomonas cepacia lipase crystals.

Figure 27 is a chromatogram illustrating the separation of a mixture of phenylacetic acid, D-4-hydroxyphenylglycine and R-phenylglycine by affinity chromatography on a chromatographic column packed with crosslinked Pseudomonas cepacia lipase crystals.

- 14 -

Figure 28 is a chromatogram illustrating the separation of a mixture of ibuprofen and L-phenyllactic acid by affinity chromatography on a chromatographic column packed with crosslinked Pseudomonas cepacia lipase crystals.

Figure 29 is a chromatogram illustrating the separation of a mixture of ibuprofen and flurbiprofen by affinity chromatography on a chromatographic column packed with crosslinked Pseudomonas cepacia lipase crystals.

Figure 30 is a chromatogram illustrating the separation of a mixture of ketoprofen, suprofen and flurbiprofen by affinity chromatography on a chromatographic column packed with crosslinked Pseudomonas cepacia lipase crystals.

Figure 31 is a chromatogram illustrating the separation of a mixture of the R and S optical isomers of methylmandelate by chiral chromatography on a chromatographic column packed with crosslinked Pseudomonas cepacia lipase crystals.

Figure 32 is a graph representing the pore size distribution of crosslinked penicillin acylase crystals useful in this invention.

Figure 33 is a graph representing the distribution coefficient as a function of the root mean square radius of PEG macromolecules run on a chromatographic column packed with crosslinked penicillin acylase crystals.

Figure 34 is a graph representing the relationship between the root mean square radius of PEG macromolecules and retention times on a chromatographic



- 15 -

column packed with crosslinked penicillin acylase crystals.

Figure 35 is a graph representing the relationship between the molecular weight of PEG  
5 macromolecules and retention times on a chromatographic column packed with crosslinked penicillin acylase crystals.

Figure 36 is a graph representing the pore size distribution of crosslinked bovine serum albumin  
10 crystals useful in this invention.

Figure 37 is a graph representing the distribution coefficient as a function of the root mean square radius of PEG macromolecules run on a chromatographic column packed with crosslinked bovine  
15 serum albumin crystals.

Figure 38 is a graph representing the relationship between the root mean square radius of PEG macromolecules and retention times on a chromatographic column packed with crosslinked bovine serum albumin  
20 crystals.

Figure 39 is a graph representing the relationship between the molecular weight of PEG macromolecules and retention times on a chromatographic column packed with crosslinked bovine serum albumin  
25 crystals.

Figure 40 is a chromatogram illustrating the separation of PEG samples by size exclusion chromatography on a chromatographic column packed with crosslinked bovine serum albumin crystals. The figure  
30 includes chromatograms of four PEG standards with average molecular weights of 62, 400, 1,500 and 10,000 daltons.

- 16 -

Figure 41 is a chromatogram illustrating the separation of a mixture of ketoprofen, suprofen and naproxen by affinity chromatography on a chromatographic column packed with crosslinked bovine serum albumin crystals.

Figure 42 is a chromatogram illustrating the separation of a mixture of the D and L optical isomers of phenyllactic acid by chiral chromatography on a chromatographic column packed with crosslinked bovine serum albumin crystals.

Figure 43 is a chromatogram illustrating the separation of PEG samples by size exclusion chromatography on a chromatographic column packed with crosslinked human serum albumin crystals. The figure includes chromatograms of two PEG standards with average molecular weights of 400 and 5,000 daltons.

Figure 44 is a chromatogram illustrating the separation of a mixture of S ibuprofen and R phenyllactic acid by adsorption chromatography on a chromatographic column packed with crosslinked thermolysin crystals (TR-2).

Figure 45 is a chromatogram illustrating the separation of a mixture of the R and S optical isomers of phenylglycine by chiral chromatography on a chromatographic column packed with crosslinked thermolysin crystals (TR-2).

Figure 46 is a chromatogram illustrating the separation of the chiral compound folinic acid on a chromatographic column packed with crosslinked human serum albumin crystals (particle size 10  $\mu$ ).

Figure 47 is a chromatogram illustrating the separation of the chiral compound 2-phenylpropionic

- 17 -

acid on a chromatographic column packed with crosslinked human serum albumin crystals.

Figure 48 is a chromatogram illustrating the separation of the chiral compound N-2,4-DNP-DL- $\alpha$ -amino-n-butyrlic acid on a chromatographic column packed with crosslinked human serum albumin crystals.

Figure 49 is a chromatogram illustrating the separation of the chiral compound N-2,4-DNP-DL-glutamic acid on a chromatographic column packed with crosslinked human serum albumin crystals.

Figure 50 is a chromatogram illustrating the separation of the chiral compound phenylglycine on a chromatographic column packed with crosslinked human serum albumin crystals.

Figure 51 is a chromatogram illustrating the separation of the chiral compound N-2,4-DNP-citrulline on a chromatographic column packed with crosslinked human serum albumin crystals.

Figure 52 is a chromatogram illustrating the separation of the chiral compound folinic acid on a chromatographic column packed with crosslinked human serum albumin crystals (particle size 25  $\mu$ ).

Figure 53 is a chromatogram illustrating the separation of the chiral compound folinic acid on a chromatographic column packed with crosslinked human serum albumin crystals (particle size 3-5  $\mu$ ).

#### DETAILED DESCRIPTION OF THE INVENTION

In order that the invention herein described may be more fully understood, the following detailed description is set forth. In the description, the following terms are employed:

- 18 -

Separation Based on Physical or Chemical Properties -- separation by means other than specific binding affinity between the active binding site of the protein component of the crosslinked protein crystals and the substance to be isolated, i.e., the substance is not a substrate, substrate analog, ligand or inhibitor of the protein. Examples of separation or purification based on physical or chemical properties include those based on molecular weight, molecular size, solubility, charge, hydrophobicity, hydrophilicity, polarity and chirality.

Separation -- Separation of a substance from a mixture of two or more different substances, i.e., substances having different physical or chemical properties, or two or more forms of the same substance. According to another embodiment of this invention, "separation" is defined as purification of a substance from a crude form thereof. Separation may be carried out by any means including, for example, chromatography, membrane separation, filtration, electrophoresis and simulated moving bed technology.

Sample -- a mixture of two or more different substances or two or more forms of the same substance. According to another embodiment of this invention, a sample may be a crude form of a substance.

Organic Solvent -- any solvent of non-aqueous origin.

Aqueous-Organic Solvent Mixture -- a mixture comprising n% organic solvent, where n is between 1 and 99 and m% aqueous, where m is 100 - n.

Crosslinked protein crystals, such as crosslinked enzyme crystals, grown from aqueous

- 19 -

solution and crosslinked with a bifunctional agent, such as glutaraldehyde, exhibit remarkable characteristics, that are superior to both soluble and conventionally immobilized enzymes [St. Clair and  
5 Navia, supra]. In addition to their stability in different environments, crosslinked protein crystals produced using CLEC® technology are also mechanically stable under extreme conditions of temperature and pH, making them excellent candidates for chromatography  
10 media.

Unlike crystals of small molecules, protein crystals are macroporous materials. On average, solvent constitutes from 30% to 65% of crystal weight [B.W. Matthews, J. Mol. Biol., 33, pp. 491-97 (1968)].  
15 The uniform solvent-filled channels traverse the body of a crystal and thus, facilitate the transport of substances in and out of the crystal. The diameter of the channels depends on the nature of the protein and its crystal form, and ranges from 20 to 100 Å, that  
20 allows for macromolecules with molecular weight up to 100,000 daltons to penetrate inside the crystals with different probability, and therefore to be separated according to their size.

Unlike the majority of current porous  
25 materials (stationary phases), such as silica, zeolites and synthetic polymers, protein crystals are asymmetric molecules made of L-amino acids and can, in principle, provide stereoselective sorption of chiral ligands. Given the fact that proteins are weak ion-exchangers  
30 with isoelectric points from 2 to 12, one can easily manipulate binding of small molecules by changing pH and buffer content of the eluent.

- 20 -

Crosslinked protein crystals are useful in all types of chromatography that use a solid as either a stationary phase or support thereof. Given their high mechanical stability, affinity and

5 enantioselectivity, crosslinked protein crystals are advantageously useful in separation of molecules via at least three different mechanisms: size exclusion, affinity and chiral. By virtue of these characteristics, crosslinked protein crystals

10 constitute universal separation media, enabling use of columns packed with the same type crystals to perform size exclusion, affinity and chiral chromatography.

Various types of chromatography may be carried out using crosslinked protein crystals. Such

15 techniques include, but are not limited to, reverse phase chromatography, high pressure liquid chromatography, low pressure liquid chromatography, gel filtration chromatography, gel permeation chromatography, batch chromatography, ion exchange

20 chromatography, elution chromatography, electrochromatography, flat-bed chromatography, thin layer chromatography, paper chromatography, simulated moving bed chromatography, column gel electrophoresis and capillary gel electrophoresis. It will be

25 appreciated by those of skill in the art that the precise parameters for these various techniques may be determined without undue experimentation.

Stationary phases comprising crosslinked protein crystals maintain their performance and

30 stability in different organic solvents, pH and salt concentrations normally used for elution. In addition, they are characterized by high throughputs, without

- 21 -

loss of resolution. Crosslinked protein crystals remain stable in the presence of acidic or alkaline solutions and are resistant to degradation by detergents and chaotropic salts. Furthermore, they  
5 retain their capture efficiency in extremes of pH and temperature.

As a result of their crystalline nature, crosslinked protein crystals achieve uniformity across the entire crosslinked crystal volume. This uniformity  
10 is maintained by the intermolecular contacts and chemical crosslinks between the protein molecules constituting the crystal lattice, even when exposed to organic or mixed aqueous-organic solvents. In such solvents, the protein molecules maintain a uniform  
15 distance from each other, forming well-defined stable pores within the crosslinked protein crystals that facilitate access of substrate to the catalyst, as well as removal of product. In these crosslinked protein crystals, the lattice interactions, when fixed by  
20 chemical crosslinks, are particularly important in preventing denaturation, especially in organic solvents or mixed aqueous-organic solvents. Crosslinked protein crystals and the constituent proteins within the crystal lattice remain monodisperse in organic  
25 solvents, thus avoiding the problem of aggregation. These features of crosslinked protein crystals contribute to their utility in separations involving harsh solvent environments that may be components of many preparative or analytical scale preparations.  
30 In addition to their activity in organic solvents and aqueous-organic solvents, crosslinked

- 22 -

protein crystals are particularly resistant to proteolysis, as well as temperature and pH extremes.

By virtue of these advantages, crosslinked protein crystals permit a major improvement in separation efficiency and economy. They provide improved results under harsh conditions or situations requiring high throughput, enabling process chemists to focus less concern on chromatography conditions.

According to one embodiment of this invention, crosslinked protein crystals are characterized by stability and integrity under elution conditions used in separations, particularly chromatography elution conditions, as compared with the soluble uncrosslinked form of the protein that is crystallized to form the protein crystals that are crosslinked. Alternatively, crosslinked protein crystals are characterized by stability and integrity in the presence of a solvent contained in the sample to be separated, as compared with the soluble uncrosslinked form of the protein that is crystallized to form the protein crystals that are crosslinked.

The crosslinked protein crystals may be used for separations involved in any number of chemical processes. Such processes include industrial and research-scale processes, such as organic synthesis of specialty chemicals and pharmaceuticals, synthesis of intermediates for the production of such products, chiral synthesis and resolution for optically pure pharmaceutical and specialty chemicals. Products which may be separated include macromolecules, such as oligomers, polymers and copolymers, small organic molecules, such as chiral organic molecules, peptides,



- 23 -

proteins, carbohydrates, nucleic acids, lipids and other chemical species. Examples of macromolecules include, flexible chain macromolecules having a molecular weight of about 10,000 daltons or less and  
5 globular proteins having a molecular weight of about 20,000 daltons or less.

Any of the above-enumerated separations may be carried out in the presence of an aqueous solvent, an organic solvent, or an aqueous-organic solvent  
10 mixture. Organic solvents may be selected from the group consisting of hydrophobic solvents, hydrophilic solvents and mixtures thereof. Examples of hydrophobic solvents include ethers, polyethers, ethers of poly(ethylene glycol), toluene, octane, isooctane,  
15 hexane and cyclohexane. Examples of hydrophilic solvents include alcohols, diols, polyols, methanol, ethanol, isopropanol, tetrahydrofuran, acetonitrile, acetone, pyridine, diethylene glycol, 2-methyl-2,4-pentanediol, poly(ethylene glycol), triethylene glycol,  
20 1,4-butanediol, 1,2-butanediol, 2,3,-dimethyl-2,3-butanediol, acetonitrile, and polyvinylpyrrolidone, or mixtures thereof.

Crosslinked protein crystals may also be used for air purification in conjunction with air  
25 filtration. For example, air may be passed through a column packed with crosslinked protein crystals to filter out any unwanted contaminants.

According to an alternate embodiment of this invention, crosslinked protein crystals are useful as  
30 separation media in devices such as, for example, sensing devices.

- 24 -

Crosslinked protein crystals useful in the methods, devices and systems of this invention may be prepared by the steps of crystallizing and crosslinking the protein, which may be carried out as described in  
5 PCT patent application WO92/02617, which is incorporated herein by reference. Alternatively, crosslinked protein crystals may be prepared as illustrated below.

Preparation of Crosslinked Protein

10 Crystals - Protein Crystallization

Protein crystals are grown by the controlled precipitation of protein out of aqueous solution or aqueous solution-containing organic solvents. Conditions to be controlled include, for example, the  
15 rate of evaporation of solvent, the presence of appropriate co-solutes and buffers, pH and temperature. A comprehensive review of the various factors affecting the crystallization of proteins has been published by McPherson, Methods Enzymol., 114, pp. 112-20 (1985).  
20 McPherson and Gilliland, J. Crystal Growth, 90, pp. 51-59 (1988) have compiled comprehensive lists of proteins and nucleic acids that have been crystallized, as well as the conditions under which they were crystallized. A compendium of crystals and  
25 crystallization recipes, as well as a repository of coordinates of solved protein and nucleic acid crystal structures, is maintained by the Protein Data Bank at the Brookhaven National Laboratory [Bernstein et al., J. Mol. Biol., 112, pp. 535-42 (1977)]. These  
30 references can be used to determine the conditions necessary for crystallization of a protein previously

- 25 -

crystallized, as a prelude to the formation of an appropriate crosslinked protein crystal, and can guide the crystallization strategy for other proteins.

Alternatively, an intelligent trial and error search  
5 strategy can, in most instances, produce suitable crystallization conditions for many proteins, provided that an acceptable level of purity can be achieved for them [see e.g., C.W. Carter, Jr. and C.W. Carter, J. Biol. Chem., 254, pp. 12219-23 (1979)].

10 The protein constituent of the crosslinked protein crystals may be any protein including, for example, an enzyme, antibody or receptor.

Proteins which may be crystallized to form crosslinked protein crystals useful in this invention  
15 include, for example, bovine serum albumin, human serum albumin, hormones, such as insulin, and immunoglobulins and their Fab fragments. Enzymes which may be crystallized to form crosslinked enzyme crystals useful in this invention include hydrolases, isomerases,  
20 lyases, ligases, transferases and oxidoreductases. Examples of hydrolases include thermolysin, elastase, esterase, lipase, nitrilase, hydantoinase, asparaginase, urease, subtilisin and other proteases and lysozyme. Examples of lyases include aldolases and  
25 hydroxynitril lyase. Examples of oxidoreductases include glucose oxidase, alcohol dehydrogenase and other dehydrogenases.

For use as crosslinked protein crystals in this invention, the large single crystals which are  
30 needed for X-ray diffraction analysis are not required. Microcrystals showers are suitable. "Microcrystals"

- 26 -

are defined as crystals which are 100 $\mu$ m or less in their largest dimension.

In general, crystals are produced by combining the protein to be crystallized with an appropriate aqueous solvent or aqueous solvent containing appropriate precipitating agents, such as salts or organics. The solvent is combined with the protein at a temperature determined experimentally to be appropriate for the induction of crystallization and acceptable for the maintenance of protein activity and stability. The solvent can optionally include co-solutes, such as divalent cations, co-factors or chaotropes, as well as buffer species to control pH. The need for co-solutes and their concentrations are determined experimentally to facilitate crystallization. In an industrial scale process, the controlled precipitation leading to crystallization can best be carried out by the simple combination of protein, precipitant, co-solutes and, optionally, buffers in a batch process. Alternative laboratory crystallization methods, such as dialysis or vapor diffusion can also be adapted. McPherson, supra, and Gilliland, supra, include a comprehensive list of suitable conditions in their reviews of the crystallization literature. Occasionally, incompatibility between the crosslinking reagent and the crystallization medium might require-exchanging the crystals into a more suitable solvent system.

Many of the enzymes for which crystallization conditions have already been described, have considerable potential as stationary phases for separations employed in industrial and laboratory

- 27 -

chemical processes and may be used to prepare crosslinked protein crystals. It should be noted, however, that the conditions reported in most of the above-cited references have been optimized to yield, in most instances, a few large, diffraction quality crystals. Accordingly, it will be appreciated by those of skill in the art that some degree of adjustment of these conditions to provide a high yielding process for the large scale production of the smaller crystals used in making crosslinked protein crystals may be necessary.

Preparation of Crosslinked Protein  
Crystals - Crosslinking of Protein Crystals

Once protein crystals have been grown in a suitable medium they can be crosslinked. Crosslinking results in stabilization of the crystal lattice by introducing covalent links between the constituent protein molecules of the crystal. This makes possible the transfer of protein into an alternate reaction environment that might otherwise be incompatible with the existence of the crystal lattice or even with the existence of intact protein. Crosslinking can be achieved by a wide variety of multifunctional reagents, including bifunctional reagents. Preferably, the crosslinking agent is glutaraldehyde. For a representative listing of other available crosslinking reagents see, for example, the 1996 catalog of the Pierce Chemical Company.

Crosslinking with glutaraldehyde forms strong covalent bonds primarily between lysine amino acid residues within and between the protein molecules in

- 28 -

the crystal lattice. The crosslinking interactions prevent the constituent protein molecules in the crystal from going back into solution, effectively insolubilizing or immobilizing the protein molecules  
5 into microcrystalline particles.

The crosslinked protein crystals are typically of a shape selected from the group consisting of plates, ellipsoids, needles, polyhedrons or rods. In addition, they typically have pores with cross-  
10 sections between about 15Å and about 100Å in length. Pore volume per crystal volume typically ranges between about 25% and about 80%. The length of the crosslinked protein crystals typically ranges between about 1 µm and 200 µm. As used herein with respect to crosslinked  
15 protein crystals, the term "length" refers to the longest dimension. In some embodiments of this invention, the crosslinked protein crystals are between about 1 µm and about 50 µm in length. The thickness of the crosslinked protein crystals typically ranges  
20 between about 1 µm and 10 µm and, in some embodiments, between about 1 µm and about 5 µm. Crosslinked protein crystals may comprise between about 20% and 80% solvent by weight.

According to one embodiment of this  
25 invention, crosslinked proteins crystals have the following characteristics:

Pore size range:	between about 20Å and about 100Å in diameter
Porosity range:	between about 0.5 and about 0.8
Pore volume:	between about 0.9 ml/g and about 4 ml/g

30

- 29 -

Pore surface areas: between about 800 m<sup>2</sup>/g and  
about 2000 m<sup>2</sup>/g

Size: between about 3 µm and about  
100 µm.

5 Preparation of Crosslinked  
Protein Crystal Stationary Phases

Crosslinked protein crystals (in slurry or  
dried form), prepared as described above, or  
commercially obtained, may be used to prepare  
10 stationary phases using conventional supports and  
packing techniques, including slurry packing. For  
example, the crosslinked protein crystals may be bound  
or linked to a solid support, included in a solid  
support, packed into a housing, such as a column or a  
15 capillary tube, layered onto beads or layered in a  
plate. The crosslinked protein crystals may be packed  
into a column for size exclusion chromatography or gel  
permeation chromatography. For ion-exchange, affinity  
or other sorption-based chromatography, the crosslinked  
20 protein crystals may be incorporated into a flat-bed,  
batch or column chromatography system. Alternatively,  
crosslinked protein crystals may be incorporated into  
or deposited on membranes, for example batch membranes,  
for use in membrane-based separations. Crosslinked  
25 protein crystals may also be included in other types of  
filtration devices.

For example, the crosslinked protein crystals  
may be packed into a standard chromatography housing,  
such as a column, having an inlet port at the top and,  
30 at the bottom, means (such as a frit, filter or disk)  
for retaining the crystals and an outlet tube. Packing

- 30 -

may be at pressures between atmospheric pressure and about 7,000 psi. The top of the column is then covered and connected to an inlet tube. Equilibration solution may then be run through the column and the pH and conductivity of the flowthrough monitored, to ensure that the media is properly equilibrated. Subsequently, a sample containing the substance to be purified or isolated is loaded onto the column. The substance to be separated is retained in the crosslinked protein crystal stationary phase, with the remaining components of the mixture being recovered in the flowthrough. The column is then subjected to washing or elution to recover the substance of interest. For example, the substance of interest may be recovered from the stationary phase by washing or elution with a gas, supercritical fluid or a solvent selected from the group consisting of aqueous solvents, organic solvents and aqueous-organic solvent mixtures. As will be appreciated by those of skill in the art, the particular eluant or washing agent will depend on the particular separation technique and substance involved.

Useful analytical chromatography columns include, for example, those having dimensions of between about 5 cm and about 25 cm in length and between about 20 mm and about 50 mm in diameter. Useful preparative chromatography columns include, for example, those having dimensions of between about 50 cm and about 150 cm in length and about 5 cm and 25 cm in diameter. Alternatively, the separation media may be packed into a capillary tube of about 50  $\mu$ m in diameter for example. Alternatively, the separation media may be layered onto a membrane of between about 100  $\mu$ m and



- 31 -

about 500  $\mu\text{m}$  in thickness, or layered onto a plate of between about 100  $\mu\text{m}$  and about 300  $\mu\text{m}$  in thickness, for example.

The chromatography columns may be made of any  
5 conventional material, preferably metal or glass. One example of an apparatus useful in this invention comprises a housing, a stationary phase comprising crosslinked protein crystals contained in the housing, means (for example, a tube) for contacting a sample  
10 with the stationary phase and means (for example, a tube) for collecting the sample after it has been contacted with the stationary phase. Conventional materials may also be used for the plate or membrane.

According to one embodiment of this  
15 invention, the crosslinked protein crystal separation media further comprises or supports a separation media selected from the group consisting of solid sorbents or soft sorbents. Such sorbents include, for example, polysaccharides, including cellulose, derivatives of  
20 cellulose, starch, dextran, dextrans, derivatives of dextrans, agar or agarose; natural polymers, synthetic polymers, such as substituted or unsubstituted polyacrylamides, polyvinyl hydrophilic polymers or polystyrene. Examples of support materials include  
25 silica, alumina, zirconia oxide, aluminosilicates, zeolites, ceramic structures, cellulose, hydrated crosslinked polymers, silica gels, Sephadex™ and derivatives of Sephadex™.

The versatility of stationary phases based on  
30 crosslinked protein crystals permits the use of chromatography arrangements using a plurality of columns, each packed with the same or different

- 32 -

crosslinked protein crystals in tandem, to separate substances by different types of chromatography at each stage. For example, a first column may trap contaminants by ion exchange chromatography and a  
5 second column may isolate the target substance by size exclusion chromatography. In such systems, the outlet of the first column is connected to the inlet of the second column and eluant from the first column runs directly into the second column. Alternatively, one or  
10 more columns packed with crosslinked protein crystals may be used in tandem with one or more conventional chromatography columns.

In order that this invention may be better understood, the following examples are set forth.  
15 These examples are for the purpose of illustration only and are not to be construed as limiting the scope of the invention in any matter.

#### EXAMPLES

Based on the high mechanical stability,  
20 affinity and enantioselectivity of crosslinked protein crystals, we packed chromatographic columns with various types of crosslinked protein crystals. More particularly, we employed crosslinked protein crystals of bovine serum albumin, human serum albumin,  
25 subtilisin, thermolysin, penicillin acylase and lipases of Candida rugosa and Pseudomonas cepacia as stationary phases for liquid chromatography.

As detailed in the examples below, we found that crosslinked protein crystals can separate  
30 polyethylene glycol (PEG) molecules, according to size,

- 33 -

by a simple isocratic mode of chromatography in aqueous and organic solutions and racemic mixtures according to their stereosymmetry. In addition, we demonstrated that crosslinked protein crystals can separate a  
5 variety of compounds according to their physical properties. Accordingly, chromatographic columns packed with the same types of crosslinked protein crystals may be used to carry out size exclusion, affinity and chiral chromatography.

10 The examples were carried out using the following reagents, processes and conditions.

Source of or Process for  
Preparation of Crosslinked Protein Crystals

15 Commercially available crosslinked protein crystals used included:

Crosslinked Pseudomonas cepacia lipase crystals, sold under the name ChiroCLEC-PC™, available from Altus Biologics, Inc. (Cambridge, Massachusetts).

20 Crosslinked Candida rugosa lipase crystals, sold under the name ChiroCLEC-CR™, available from Altus Biologics, Inc. (Cambridge, Massachusetts).

Crosslinked subtilisin crystals, sold under the name ChiroCLEC-BL™, available from Altus Biologics,  
25 Inc. (Cambridge, Massachusetts).

Crosslinked thermolysin crystals, sold under the name PeptiCLEC-TR™, available from Altus Biologics, Inc. (Cambridge, Massachusetts). Starting from a batch of PeptiCLEC-TR™, we separated crystals by size. "TR-  
30 2" crystals were those having an average length of 7 µm. "TR-3" crystals were those having an average length of 30 µm.

- 34 -

Crosslinked penicillin acylase crystals, sold under the name ChiroCLEC-EC™, available from Altus Biologics, Inc. (Cambridge, Massachusetts).

5 Crystallization of Bovine Serum Albumin

- One hundred twenty grams of powdered bovine serum albumin was added to a 40 ml stirred solution of 100 mM phosphate buffer (pH 5.3) at 4°C. Final protein concentration was 250 mg/ml (estimated from OD<sub>280</sub>).
- 10 Saturated ammonium sulfate solution (767 g/l) prepared in deionized water was added to the protein solution to a final concentration of 50% saturation (350 g/l). The crystallization solution was "seeded" with 5 ml of albumin crystals (200 mg/ml) in 50% ammonium sulfate
- 15 (pH 5.3). Seed crystals were prepared by washing a sample of crystals free of precipitate with a solution of 50% saturated ammonium sulfate and 100 mg/ml soluble BSA in 100 mM phosphate buffer pH 5.3. The seeded crystallization solution was incubated at 4°C overnight
- 20 on a rotating platform. Crystal plates (20-100 µ) appeared in the solution overnight (16 hr).

Crystallization of Human Serum Albumin

- Ten grams of powdered human serum albumin was added to a 75 ml stirred solution of 100 mM phosphate
- 25 buffer (pH 5.5) at 4°C. Final protein concentration was 120 mg/ml (estimated from OD<sub>280</sub>). Saturated ammonium sulfate solution (767 g/l) prepared in deionized water was added to the protein solution to a final concentration of 50% saturation (350 g/l). The
- 30 crystallization solution was "seeded" with 1 ml of albumin crystals (50 mg/ml) in 50% ammonium sulfate

- 35 -

(pH 5.5). Seed crystals were prepared by washing a sample of crystals free of precipitate with a solution of 50% saturated ammonium sulfate in 100 mM phosphate buffer pH 5.5. The seeded crystallization solution was  
5 incubated at 4°C overnight on a vigorously rotating platform. Crystals rods (20  $\mu$ ) appeared in the solution overnight (16 hr).

#### Crosslinking of Serum Albumin Crystals

The crosslinking procedure used involved  
10 conditions designed not to disrupt crystal structure. The conditions were those which allowed crosslinking of the crystals, to prevent their dissolution in harsh conditions and render them sufficiently rigid for the purpose of chromatography.

15 Crosslinking was performed in an identical manner for the crystal solutions of each of bovine and human serum albumin. Crosslinking was performed at 4°C in a stirred solution of crystals and mother liquor containing 50% saturated ammonium sulfate as described  
20 above. The crystals were not washed prior to crosslinking with borate-pretreated glutaraldehyde.

Pretreated glutaraldehyde was prepared by adding one volume of 50% glutaraldehyde ("GA") to an equal volume of 300 mM sodium borate (pH 9). The  
25 glutaraldehyde solution was incubated at 60°C for 1 hour. The pH of the solution was then adjusted to 5.5 with concentrated HCl and the solution was rapidly cooled to 4°C (on ice).

The pretreated glutaraldehyde (25%) was added  
30 to the crystallization solution stepwise, in 0.05% increments (total concentration) at 15 minute intervals

- 36 -

to a concentration of 2%. Aliquots of crystallization solution used ranged between 1 ml and 500 ml volume. The crystals were then brought to 5% GA and incubated at 4°C for 4 hours to allow crosslinking. Albumin crosslinked crystals were collected by low speed centrifugation and washed repeatedly with pH 7.5, 100 mM Tris HCl. Washing was stopped when the crosslinked crystals could be centrifuged at high speed without aggregation.

#### 10 Chromatographic Conditions - Examples 1-15

The crosslinked crystals described above were used to pack standard chromatographic columns having the following dimensions: 25 cm x 4.6 mm, 10 cm x 4.6 mm, and 15 cm x 2.1 mm (Alltech, Waters or Hewlett Packard). Packing was carried out by means of a slurry packing method, using deionized water as packing fluid, at a pressures up to 7000 psi and a slurry packer machine (Alltech, Catalog No. 1666). The loaded columns remained useful for 4 months (up to 1000 injections).

A liquid chromatograph, HP-1050, coupled with two detectors on line, UV and RI, was used in the chromatographic experiments. Sodium phosphate buffer with different pH values was used as a solvent for the affinity and chiral chromatography experiments. The best resolution for phenyllactic acid isomers was obtained at pH 7.5, flow rate 0.2 ml/min and injection volume 1.5 µl, with detection by UV at 218 nm. The solute concentration was about 5 mg/ml.

Affinity chromatography was performed under the same conditions, but using a flow rate of 0.5

- 37 -

ml/min. The solute concentration was about 0.2 mg/ml and injection volume was 20  $\mu$ l.

Size exclusion chromatography of various PEG samples was performed, first, in the same sodium phosphate buffer with pH 8.3, second, in deionized water, and third, in 15% and 50% of aqueous acetonitrile solution, with a flow rate of 0.5 ml/min and detection by means of RI and UV at 192 nm. The concentration of the PEG solution was about 3 mg/ml.

The following table summarizes the specific experimental conditions for experiments, including those detailed in the examples. In the table, the following abbreviations are used, in addition to "TR-2" and "TR-3" as defined above:

"CR" - Candida rugosa lipase  
 "PC" - Pseudomonas cepacia lipase  
 "BSA" - Bovine serum albumin  
 "HSA" - Human serum albumin  
 "PA" - Penicillin acylase.

Protein Crystal	Column Size	Pressure at Packing (psi)	Amount of packed crystals (g)	Flow rate (ml/min)	Pressure Drop (psi)	pH	Eluent
TR-3	25cm x 4.6mm	1500	1	0.2 - 0.5	180 - 600	5 - 8.5	TRIS, H <sub>2</sub> O, 15% CH <sub>3</sub> CN 50% CH <sub>3</sub> CN
TR-2	25cm x 4.6mm	2500	1	0.2 - 0.5	180 - 600	5 - 8.5	TRIS, H <sub>2</sub> O, 15% CH <sub>3</sub> CN 50% CH <sub>3</sub> CN
CR	25cm x 4.6mm	2000	1	0.2 - 0.5	180 - 600	5 - 8.5	TRIS, H <sub>2</sub> O, 15% CH <sub>3</sub> CN 50% CH <sub>3</sub> CN

- 38 -

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Protein Crystal	Column Size	Pressure at Packing (psi)	Amount of packed crystals (g)	Flow rate (ml/min)	Pressure Drop (psi)	pH	Eluent
PC	10cm x 4.6mm	2500	0.5	0.2 - 0.5	180 - 600	5 - 8.5	TRIS, H <sub>2</sub> O, 15% CH <sub>3</sub> CN 50% CH <sub>3</sub> CN
BSA	25cm x 4.6mm	7000	1	0.2 - 0.5	180 - 600	5 - 8.5	TRIS, H <sub>2</sub> O, 15% CH <sub>3</sub> CN 50% CH <sub>3</sub> CN
HSA	15cm x 2.1mm	4000	0.2	0.2 - 0.5	180 - 600	5 - 8.5	TRIS, H <sub>2</sub> O, 15% CH <sub>3</sub> CN 50% CH <sub>3</sub> CN
PA	10cm x 4.6mm	2500	0.5	0.2 - 0.5	180 - 600	5 - 8.5	TRIS, H <sub>2</sub> O, 15% CH <sub>3</sub> CN 50% CH <sub>3</sub> CN

5

### Porosimetry

Pore size, pore size distribution and porosity of the crosslinked protein crystals was determined by macromolecular porosimetry [L.Z. Vilenchik et al., "Macromolecular Porosimetry", J. Chromatogr., 648, pp. 9-17 (1993); J.H. Knox and H.P. Scott, J. Chromatogr., 316, p. 311 (1984)]. That technique permits determination of such characteristics for solid and soft porous sorbents by measuring a coefficient of distribution (Kd) for macromolecules in a size exclusion chromatography process. Kd, which represents the distribution of macromolecules between pore volume and interparticle volume, is a function of molecular size, pore size and molecular distribution.

As described in the examples below, crosslinked protein crystals are extremely stable, have porous structure and exhibit great affinity and chiral selectivity. In essence, crosslinked protein crystals meet all the major requirements for chromatographic



- 39 -

stationary phases. For example, their pore size is comparable to that of the molecules being analyzed. The property permits analysis of flexible chain macromolecules in the range of molecular weight up to 10,000 daltons and globular molecules up to 20,000 daltons. From the same data, porosity of the crosslinked protein crystals, i.e., pore volume per crystal volume is close to 50%, a level attractive for chromatographic stationary phases. By fractionating the crystals, it is possible to obtain narrow fractions according to their size to achieve high efficiency for chromatographic columns packed with them.

Such properties establish crosslinked protein crystals as a new generation of nanoporous materials that can perform as a separation media suitable for different types of chromatography and simulating moving bed technology [M.J. Gattuso et al., "Simulated Moving Bed Technology -- The Preparation of Single Enantiomer Drugs", Pharmaceutical Technology Europe, pp. 20-25 (June 1996)].

In various of the chromatograms discussed below, the retention time for each compound is indicated on the peak relating to that compound.

#### Example 1

A chromatographic column was packed with TR-3 crosslinked thermolysin crystals, prepared as described above, by means of a slurry packing method using deionized water as packing fluid at a pressure of 1500 psi. An aliquot of about 1 g of the crystals was packed into the column, which was 25 cm x 4.6 mm in size. The PEG standards were dissolved in water. Concentration of the solutions was 3 mg/ml. The

- 40 -

temperature was ambient. Injection volume was 25  $\mu$ l, flow rate 0.5 ml/min.

Figure 1 shows pore size distribution in the TR-3 crystals. Figure 2 shows the distribution coefficient for PEG macromolecules inside the TR-3-packed column with as a function of root mean square radius of the macromolecules. Figures 3 and 4 show raw chromatographic data for the PEG macromolecules run on the chromatographic column. The data were used to calculate the distribution coefficient shown in Figure 2 and that coefficient was used to calculate the pore size distribution in Figure 1 using the macromolecular porosimetry technique.

#### 15 Example 2

Figures 5 and 6 show separation of PEG samples according to size of their macromolecules by means of size exclusion chromatography on the TR-3-crystal packed column described in Example 1.

20 Figure 5 shows separation of a mixture of three PEG standards in the chromatography run (RI detection). They were: PEG with average molecular weights of 62, 600 and 10,000 daltons. Tris buffer 10 mM (pH 7.4) was used as a solvent. Flow rate was 25 0.5 ml/min. Injection volume was 20  $\mu$ l. The pressure drop inside the column during the chromatographic run was 220 psi. Concentrations of the PEG samples used to prepare the mixture were 3 mg/ml.

Figure 6 shows the overlaid chromatograms (RI detection) of six PEG standards with molecular weights of 62, 106, 194, 400, 1,500 and 10,000 daltons. They were run on the same column that was used to generate

- 41 -

Figure 5, except that the solvent employed was 50% of  $\text{CH}_3\text{CN}$  + 50% of  $\text{H}_2\text{O}$ . That solvent was used in order to demonstrate the ability of the column to perform properly in the presence of organic solvent. pH value  
5 of the solvent was 7.0, flow rate was 0.5 ml/min.

### Example 3

Figures 7-10 show application of TR-3 crosslinked thermolysin crystals for affinity chromatography. Figures 7 and 8 (UV detection,  
10  $\lambda=218\text{nm}$ ) demonstrate separation of substances according to their chemical nature. The substances were phenylacetic acid, hydroxyphenylglycine and R-phenylglycine (in Figure 7) and phenylacetic acid, hydroxyphenylglycine, R-phenylglycine and L--  
15 phenylalaninamide (in Figure 8). These substances are not substrates of thermolysin. The chromatography was carried out in Tris buffer 10 mM (pH 8.4) at a flow rate of 0.5 ml/min.

Figure 9 (UV detection,  $\lambda=218\text{nm}$ ) shows  
20 separation according to chemical structure of a mixture of D-isomer of phenyllactic acid and ibuprofen on the TR-3-packed column. The solvent was Tris buffer 10 mM (pH 8.15). Flow rate was 0.5 ml/min.

Figure 10 is a chromatogram (RI detection) of  
25 flurbiprofen on the TR-3-packed column. Figure 11 demonstrates separation according to chemical structure of a mixture of ibuprofen (two peaks) and flurbiprofen (RI detection). In the separations depicted in Figures 10 and 11, the solvent was Tris buffer 10 mM (pH 7.5)  
30 and the flow rate was 0.5 ml/min.

- 42 -

Example 4

Example 4 shows the application of TR-2 and TR-3 crosslinked thermolysin crystals, prepared as described above, for chiral chromatography.

5           Figure 12 demonstrates chiral separation of two optical isomers of phenyllactic acid R and S on two columns: one packed with TR-3 and the other packed with TR-2. The solvent was Tris buffer 10 mM (pH 7.5), the flow rate was 0.2 ml/min and the injection volume was  
10 10  $\mu$ l (UV detection  $\lambda=218\text{nm}$ ). The pressure drop inside the columns at the chromatographic run was 285 psi.

          Figure 13 shows the same separation, using only the TR-2-packed column but with an injection volume of 1.5  $\mu$ l (UV detection,  $\lambda=218\text{nm}$ ). Flow rate  
15 was 0.3 ml/min. The pressure drop inside the column at the chromatographic run was 500 psi.

          Figures 14 and 15 show a chiral separation of two optical isomers of phenylglycine (R and S) in a chromatographic system using two columns: TR-3-packed  
20 and TR-2-packed, with an injection volume of 10  $\mu$ l (Figure 14) and 1.5  $\mu$ l (Figure 15), respectively. In the separations, the solvent was Tris buffer 10 mM (pH 7.5), flow rate was 0.2 ml/min and the pressure drop inside the columns at the chromatographic runs was 285  
25 psi (UV detection,  $\lambda=218\text{nm}$ ).

Example 5

          A chromatographic column was packed with ChiroCLEC-CR<sup>TM</sup> crystals by means of a slurry packing method using deionized water as packing fluid at a  
30 pressure of 2000 psi. An aliquot of about 1 g of the crystals was packed into the column, which was

- 43 -

25 cm x 4.6 mm in size. The PEG standards were dissolved in water. Concentration of the solutions was 3 mg/ml. The temperature was ambient. Injection volume was 25  $\mu$ l, flow rate 0.5 ml. Figure 16 shows pore size distribution in the ChiroCLEC-CR<sup>TM</sup> crystals. Figure 17 shows the distribution coefficient for PEG macromolecules inside the ChiroCLEC-CR<sup>TM</sup>-packed column, as a function of root mean square radius of the macromolecules. Figures 18 and 19 show raw chromatographic data for the PEG macromolecules run on the chromatographic column. The data were used to calculate the distribution coefficient shown in Figure 17 and that coefficient was used to calculate the pore size distribution in Figure 16 by means of macromolecular porosimetry.

#### Example 6

Figures 20, 21 and 22 (RI detection), show separation of PEG samples according to size of their macromolecules by means of size exclusion chromatography on the column packed with ChiroCLEC-CR<sup>TM</sup> crystals, as described in Example 5.

Figure 20 shows the overlaid chromatograms (RI detection) of four PEG standards with molecular weights of 62, 400, 1,500, and 10,000 daltons.

The solvent used was Tris buffer 10 mM (pH 8), flow rate was 0.5 ml/min, pressure drop inside the column at the chromatographic run was 307 psi.

Figure 21 shows PEG separation on the same ChiroCLEC-CR<sup>TM</sup>-packed column. However, the solvent used was 50% of CH<sub>3</sub>CN + 50% of H<sub>2</sub>O, in order to demonstrate ability of the column to perform properly at the

- 44 -

presence of organic solvent. The pH value of the solvent was 7.0, flow rate was 0.5 ml/min and the pressure drop inside the column at the chromatographic run was 260 psi. The overlaid chromatograms in Figure 21 represent the following PEG samples: Mw = 62, 106, 194, 410, 1,500 and 10,000 daltons.

Figure 22 shows PEG separation on the same ChiroCLEC-CR<sup>TM</sup>-packed column using 15% of CH<sub>3</sub>CN + 50% of H<sub>2</sub>O as solvent. The pH value of the solvent was 7.0, flow rate was 0.5 ml/min and the pressure drop inside the column at the chromatographic run was 296 psi. The overlaid chromatograms in Figure 22 represent the following PEG samples: Mw = 194, 900, 1,500 and 5,000 daltons.

#### 15 Example 7

A chromatographic column was packed with ChiroCLEC-PC<sup>TM</sup> crystals by means of a slurry packing method using deionized water as packing fluid at a pressure of 2500 psi. An aliquot of about 0.5 g of the crystals was packed into the column, which was 10cm x 4.6mm in size. The PEG standards were dissolved in water. Concentration of the solutions was 3 mg/ml. The temperature was ambient. Injection volume was 25 µl, flow rate 0.5 ml.

Figure 23 shows pore size distribution in the ChiroCLEC-PC<sup>TM</sup> crystals. Figure 24 shows the distribution coefficient for PEG macromolecules inside the column with ChiroCLEC-PC<sup>TM</sup> crystals as a function of root mean square radius of the macromolecules. Figures 25 and 26 show raw chromatographic data for the PEG macromolecules run on the chromatographic column. The

- 45 -

data were used to calculate the distribution coefficient shown in Figure 24 and that coefficient was used to calculate the pore size distribution in Figure 23 by macromolecular porosimetry.

5

#### Example 8

Figures 27-30 (UV detection,  $\lambda=215\text{nm}$ ) show application of ChiroCLEC-CR<sup>TM</sup> crystals for affinity chromatography to separate substances according to their chemical nature using the column described in Example 7. The substances were: phenyllactic acid, R-phenylglycine and D-4-hydroxyphenylglycine in Figure 27; ibuprofen and phenylacetic acid-dl in Figure 28; ibuprofen and flurbiprofen in Figure 29; suprofen, ketoprofen and flurbiprofen in Figure 30. In each chromatography, Tris buffer 10 mM (pH 7.4) was used as solvent and flow rate was 0.5 ml/min.

#### Example 9

This example shows the application of ChiroCLEC-PC<sup>TM</sup> crystals for chiral chromatography using the same column and chromatographic conditions as in Examples 7 and 8.

Figure 31 demonstrates a chiral separation of two optical isomers of methylmandelate, R and S on the ChiroCLEC-PC<sup>TM</sup> crystal-packed column (RI detection).

#### Example 10

A chromatographic column was packed with ChiroCLEC-EC<sup>TM</sup> crystals by means of a slurry packing method using deionized water as packing fluid at a pressure of 2500 psi. An aliquot of about 0.5 g of the

- 46 -

crystals was packed into the column, which was 10 cm x 4.6 mm in size. The PEG standards were dissolved in water. Concentration of the solutions was 3 mg/ml. The temperature was ambient. Injection volume was  
5 25  $\mu$ l and flow rate 0.5 ml.

Figure 32 shows pore size distribution in the penicillin acylase crystals. Figure 33 shows the distribution coefficient for PEG macromolecules inside the column with ChiroCLEC-EC<sup>TM</sup> crystals as a function of  
10 root mean square radius of the macromolecules. Figures 34 and 35 show raw chromatographic data for the PEG macromolecules run on the chromatographic column. The data were used to calculate the distribution coefficient shown in Figure 33 and that coefficient was  
15 used to calculate the pore size distribution in Figure 32 by means of macromolecular porosimetry.

#### Example 11

A chromatographic column was packed with bovine serum albumin crosslinked protein crystals,  
20 prepared as described above, by means of a slurry packing method using deionized water as packing fluid at a pressure of 7000 psi. An aliquot of about 1 g of the crystals was packed into the column, which was 25 cm x 4.6 mm in size. The PEG standards were dissolved  
25 in water. Concentration of the solutions was 3 mg/ml. The temperature was ambient. Injection volume was 25  $\mu$ l, flow rate 0.5 ml.

Figure 36 shows pore size distribution in the BSA crystals. Figure 37 shows the distribution  
30 coefficient for PEG macromolecules inside the column with BSA crystals as a function of root mean square



- 47 -

radius of the macromolecules. Figures 38 and 39 show raw chromatographic data for the PEG macromolecules run on the chromatographic column. The data were used to calculate the distribution coefficient shown in Figure 37 and that coefficient was used to calculate the pore size distribution in Figure 36 by means of macromolecular porosimetry.

#### Example 12

10               Figure 40 shows separation of PEG samples according to size of their macromolecules by means of size exclusion chromatography on the column packed with bovine serum albumin crosslinked protein crystals, as described in Example 11.

15               Figure 40 shows the overlaid chromatograms (RI detection) of four PEG standards with  $M_w = 62, 400, 1,500$  and  $10,000$  daltons. The solvent used was Tris buffer (pH 7.2), flow rate was  $0.5 \text{ ml/min}$  and the pressure drop inside the column at slurry packing was  
20   210 psi.

#### Example 13

              Figure 41 shows application of bovine serum albumin crosslinked protein crystals for affinity chromatography (UV detection,  $\lambda=256\text{nm}$ ). Figure 41  
25 demonstrates the overlaid chromatograms of ketoprofen, suprofen and naproxen. The solvent used was Tris buffer (pH 8.2), flow rate was  $0.5 \text{ ml/min}$  and the pressure drop inside the column at the chromatographic run was 280 psi.

- 48 -

Example 14

This example shows the application of bovine serum albumin crosslinked protein crystals for chiral chromatography.

- 5           Figure 42 demonstrates a chiral separation of two optical isomers of phenyllactic acid, D and L, on a BSA-packed column. The column and the chromatographic conditions were the same as in Example 13 (RI detection).

10 Example 15

- A chromatographic column was packed with human serum albumin crosslinked protein crystals by means of a slurry packing method using deionized water as packing fluid at a pressure of 4000 psi. An aliquot  
15 of about 0.2 g of the crystals was packed into the column, which was 15 cm x 2.1 mm in size. The PEG standards were dissolved in water. Concentration of the solutions was 3 mg/ml. The temperature was ambient. Injection volume was 25  $\mu$ l, flow rate  
20 0.15 ml/min.

- Figure 43 shows the size exclusion separation of two PEG samples ( $M_w = 400$  and 5,000 daltons) on the column packed with HSA crosslinked protein crystals. The solvent used was Tris buffer 10 mM (pH 7.5), flow  
25 rate was 0.15 ml/min and the pressure drop inside the column at the chromatographic run was 790 psi (RI detection).

- 49 -

Example 16

This example shows the application of TR-2 crosslinked thermolysin crystals, prepared as described above, for adsorption chromatography.

5                Figure 44 demonstrates separation of S ibuprofen and R phenyllactic acid on a column packed with TR-2. The solvent was Tris buffer 10 mM (pH 8.15), the flow rate was 0.5 ml/min, the sample concentration was 0.2 mg/ml and the injection volume  
10 was 20  $\mu$ l (UV detection  $\lambda$ =218nm).

Example 17

This example shows the application of TR-2 crosslinked themolysin crystals, prepared as described above, for chiral chromatography.

15                Figure 45 demonstrates chiral separation of two optical isomers of phenylglycine R and S on a column packed with TR-2. The solvent was Tris buffer 10 mM (pH 7.5), the flow rate was 0.5 ml/min, the sample concentration was 0.2 mg/ml and the injection  
20 volume was 20  $\mu$ l (UV detection  $\lambda$ =218nm).

Example 18

Human serum albumin crosslinked crystals, prepared as described above, were used to carry out chiral chromatography of various chiral compounds.

25 Crosslinked human serum albumin crystals-were mixed with silica in the proportion specified below for each chromatography. The mixture was then filtered through a 1  $\mu$  filter to remove fines. First, aqueous slurry containing 50 mg pure silica was packed into each  
30 column, followed by the silica/crosslinked crystal

- 50 -

mixture using a slurry packing machine (Alltech) under packing pressure below 2,000 psi. Packing time was 30 to 40 minutes. The details of each separation are listed below:

5 Folinic Acid

	Column:	16% crosslinked HSA crystals and 84% silica gel in a 50 x 4 mm column.
10	Mobile phase:	100 mM sodium phosphate buffer (pH 6.9).
	Flow rate:	0.7 ml/min, pressure 90 bar.
	Detector:	UV 225 nm.
	Sample concentration:	1.2 mg/ml.
15	Injection volume:	0.5 $\mu$ l.

2-Phenylpropionic Acid

	Column:	16% crosslinked HSA crystals and 84% silica gel in a 50 x 4.6 mm column.
20	Mobile phase:	3% 2-propanol in 0.1 M sodium phosphate buffer (pH 6.3).
	Flow rate:	0.7 ml/min, pressure 90 bar.
	Detector:	UV 225 nm.
25	Sample concentration:	0.4 mg/ml.

N-2,4-DNP-DL- $\alpha$ -amino-n-butyric acid

- 51 -

Column: 16% crosslinked HSA crystals  
and 84% silica gel in a  
50 x 4.6 mm column.

5 Mobile phase: 13% 1-propanol in 0.01 M  
sodium phosphate buffer (pH  
7.0).

Flow rate: 0.7 ml/min, pressure 188 bar.

Detector: UV 210 nm.

10 Sample  
concentration: 1.2 mg/ml.

N-2,4-DNP-Glutamic Acid

Column: 16% crosslinked HSA crystals  
and 84% silica gel in a  
50 x 4 mm column.

15 Mobile phase: 10% 1-propanol in 100 mM  
sodium phosphate buffer  
(pH 6.9).

Flow rate: 0.7 ml/min, pressure 90 bar.

Detector: UV 225 nm.

20 Sample  
concentration: 0.6 mg/ml.

Injection volume: 0.5 µl.

Phenylglycine

25 Column: 14% crosslinked HSA crystals  
and 86% silica gel in a  
100 x 4.6 mm column.

Mobile phase: 3% 2-propanol in 0.1 M sodium  
phosphate buffer (pH 6.0).

Flow rate: 0.7 ml/min.

30 Detector: UV 225 nm.

- 52 -

Sample  
concentration: 2 mg/ml.

N-2,4-DNP-Citrulline

5           Column:           16% crosslinked HSA crystals  
                                  and 84% silica gel in a  
                                  50 x 4.6 mm column.

          Mobile phase:       10% 2-propanol in 0.01 M  
                                  sodium phosphate buffer  
                                  (pH 7.0).

10          Flow rate:         0.5 ml/min, pressure 190 bar.

          Detector:          UV 210 nm.

          Sample  
          concentration:       1.2 mg/ml.

          Injection volume:   0.5 µl.

15               Figures 46-51 depict these chiral  
                  separations.

Example 19

                  This example shows the correlation between  
                  separation efficiency and particle size of crosslinked  
20   human serum albumin crystals, prepared as described  
                  above, in the chiral chromatography of the chiral  
                  compound folinic acid.

                  The results are depicted in the  
                  chromatographs of Figures 46 and 52-53 for,  
25   respectively, crosslinked HSA crystals of 25 µ average  
                  length (Figure 52); crosslinked HSA crystals crushed by  
                  ultrasound -- 10 µ average length (Figure 46) and  
                  crosslinked HSA crystals which are small uniform

- 53 -

particles of 3-5  $\mu$  (Figure 53). The conditions of these chromatographies were as follows:

5	Column:	16% crosslinked HSA crystals and 84% silica gel in a 50 x 4 mm column.
	Mobile phase:	100 mM sodium phosphate buffer (pH 6.9).
	Flow rate:	0.7 ml/min, pressure 90 bar.
	Detector:	UV 225 nm.
10	Sample concentration:	1.2 mg/ml.
	Injection volume:	0.5 $\mu$ l.

#### Example 20

Example 20 shows the stability of crosslinked human serum albumin crystals, prepared as described above, toward organic solvents.

A standard 10 cm chromatography column was packed with 140 mg crosslinked human serum albumin crystals and 860 mg silica. The column was then washed with acetonitrile for 75 minutes. The capacity factors and selectivity of the separation of folinic acid remained almost identical before ( $k_1$  2.3,  $k_2$  18.8,  $\alpha$ =8.2) and after ( $k_1$  2.8,  $k_2$  18.1,  $\alpha$ =6.5) washing. This indicates a high level of stability of the crosslinked human serum albumin crystals under chromatographic conditions.

#### Example 21

- 54 -

This example shows the high loading capacity of crosslinked human serum albumin crystals as prepared above in the resolution of folinic acid.

A standard column (100 x 4.6 mm) was packed  
5 with a slurry of 14% crosslinked human serum albumin crystals and 86% silica. The mobil phase was 4% 2-propanol in 0.1 M phosphate buffer (pH 7). The flow rate was 0.5 ml/min. The capacity factor  $k_1 = (v_1 - v_0)/v_0$ ; selectivity  $\alpha = k_2/k_1 = (v_2 - v_0)/(v_1 - v_0)$ . As  
10 shown in the table below, as progressively increasing amounts of folinic acid were injected into the column, the selectivity of the separation remained good.

	Folinic acid (mg)	HSA (mg)	Capacity Factor $k_1$	Capacity Factor $k_2$	Selectivity $\alpha$	Loading mg/g HSA
15	0.064	140	1.8	9.0	4.9	0.46
	0.128	140	1.7	7.6	4.5	0.92
	0.192	140	1.5	6.8	4.4	1.37
	0.320	140	1.5	4.0	2.7	2.29

While we have hereinbefore described a number  
20 of embodiments of this invention, it is apparent that our basic constructions can be altered to provide other embodiments which utilize the processes and compositions of this invention. Therefore, it will be appreciated that the scope of this invention is to be  
25 defined by the claims appended hereto rather than by the specific embodiments which have been presented hereinbefore by way of example.



- 55 -

### CLAIMS

We Claim:

1. A method for separation of a substance from a sample comprising the step of contacting said sample with a separation media, said separation media comprising crosslinked protein crystals, for a sufficient time and under conditions which permit said substance to be retained by said separation media based on a physical or chemical property of that substance.
2. The method according to claim 1, further comprising the step of separating said substance from said separation media.
3. The method according to claim 1, wherein said substance is retained within said separation media based on a physical property of the substance selected from the group consisting of molecular weight, molecular size, solubility, charge, hydrophobicity, hydrophilicity, polarity and chirality.
4. The method according to claim 1, wherein said separation is carried out by chromatography.
5. The method according to claim 4, wherein said chromatography is selected from the group consisting of adsorption chromatography, size exclusion chromatography and chiral chromatography.
6. The method according to claim 5, wherein said chromatography is selected from the group

- 56 -

consisting of thin layer chromatography, gel permeation chromatography, reverse phase chromatography, column chromatography, batch chromatography, ion-exchange chromatography, paper chromatography, flat bed chromatography, simulated moving bed chromatography, column electrophoresis and capillary electrophoresis.

7. The method according to claim 1, wherein said separation media is in a stationary phase.

8. The method according to claim 1, wherein said separation media is in a moving bed or a fluidized bed.

9. The method according to claim 1, wherein said sample comprises or is contained in a liquid mobile phase, a gas phase or a supercritical fluid phase.

10. The method according to claim 2, wherein said substance is separated from said separation media by elution.

11. The method according to claim 10, wherein said substance is separated from said separation media by elution with a solvent selected from the group consisting of aqueous solvents, organic solvents and aqueous-organic solvent mixtures.

12. The method according to claim 10, wherein said substance is separated from said

- 57 -

separation media by elution with a gas or a supercritical fluid.

13. The method according to claim 1, wherein said separation media further comprises or supports a separation media selected from the group consisting of solid sorbents or soft sorbents.

14. The method according to claim 13, wherein said solid sorbent or soft sorbent separation media is selected from the group consisting of silica gels, porous glass, beads, silica, alumina, zirconia oxide, aluminosilicates, zeolites, ceramics, polysaccharides, dextrans, derivatives of dextrans, cellulose, derivatives of cellulose, synthetic polymers, natural polymers and hydrated crosslinked polymers.

15. The method according to claim 1, wherein said crosslinked protein crystals are microcrystals.

16. The method according to claim 11, wherein said crosslinked protein crystals are characterized by stability and integrity under chromatographic elution conditions as compared with the soluble uncrosslinked form of the protein that is crystallized to form said protein crystals that are crosslinked.

17. The method according to claim 1, wherein said protein is selected from the group consisting of enzymes, antibodies and receptors.

- 58 -

18. The method according to claim 1, wherein said protein is selected from the group consisting of human serum albumin and bovine serum albumin.

19. The method according to claim 17, wherein said protein is an enzyme selected from the group consisting of hydrolases, isomerases, lyases, ligases, transferases and oxidoreductases.

20. The method according to claim 19, wherein said enzyme is a hydrolase.

21. The method according to claim 20, wherein said hydrolase is selected from the group consisting of thermolysin, elastase, esterase, lipase, nitrilase, hydantoinase, protease, asparaginase, subtilisin, penicillin acylase, urease and lysozyme.

22. The method according to claim 21, wherein said lipase is selected from the group consisting of Candida rugosa lipase and Pseudomonas cepacia lipase.

23. The method according to claim 1, wherein said sample comprises or is contained in a solvent selected from the group consisting of aqueous solvents, organic solvents and aqueous-organic solvent mixtures.

24. The method according to claim 23, wherein said organic solvent is selected from the group consisting of hydrophobic solvents, hydrophilic solvents and mixtures thereof.

- 59 -

25. The method according to claim 23, wherein said crosslinked protein crystals are characterized by stability and integrity in the presence of said solvent as compared with the soluble uncrosslinked form of the protein that is crystallized to form said protein crystals that are crosslinked.

26. The method according to claim 1, wherein said substance is selected from the group consisting of macromolecules, small organic molecules, peptides, proteins, carbohydrates, nucleic acids and lipids.

27. The method according to claim 26, wherein said small organic molecules are chiral organic molecules.

28. The method according to claim 26, wherein said macromolecules are selected from the group consisting of oligomers, polymers and copolymers.

29. The method according to claim 28, wherein said macromolecules are selected from the group consisting of flexible chain macromolecules having a molecular weight of about 10,000 daltons or less and globular proteins having a molecular weight of about 20,000 daltons or less.

30. The method according to claim 1, wherein said crosslinked protein crystals are bound to or included in a solid support.

- 60 -

31. The method according to claim 1, wherein said crosslinked protein crystals are packed in a column, layered in a plate or included in a membrane.

32. The method according to claim 1, wherein said crosslinked protein crystals are in a form selected from the group consisting of plates, ellipsoids, needles, polyhedrons and rods.

33. The method according to claim 1, wherein said crosslinked protein crystals have pores with cross-sections between about 15Å and about 100Å in length.

34. The method according to claim 1, wherein said crosslinked protein crystals comprise between about 20% and 80% solvent by weight.

35. The method according to claim 1, wherein said crosslinked protein crystals are between about 1  $\mu\text{m}$  and about 200  $\mu\text{m}$  in length.

36. The method according to claim 35, wherein said crosslinked protein crystals are between about 1  $\mu\text{m}$  and about 50  $\mu\text{m}$  in length.

37. The method according to claim 1, wherein said crosslinked protein crystals are between about 1  $\mu\text{m}$  and about 10  $\mu\text{m}$  in thickness.

- 61 -

38. The method according to claim 37, wherein said crosslinked protein crystals are between about 1  $\mu\text{m}$  and about 5  $\mu\text{m}$  in thickness.

39. The method according to claim 1, wherein said crosslinked protein crystals have a pore volume per crystal volume between about 25% and about 80%.

40. An apparatus for carrying out a separation, comprising:

(a) separation media comprising crosslinked protein crystals; and

(b) means for contacting a sample with said separation media.

41. The apparatus according to claim 40, further comprising means for separating said substance from said separation media.

42. The apparatus according to claim 40, wherein said separation media is in a stationary phase.

43. The apparatus according to claim 40, wherein said separation is chromatography.

44. The apparatus according to claim 41, wherein said means for contacting said sample with said separation media is selected from the group consisting of columns, membranes, plates, batches and capillary tubes.

- 62 -

45. The apparatus according to claim 44, wherein said separation media is packed into said column.

46. The apparatus according to claim 45, wherein said column has dimensions of between about 5cm and about 25 cm in length and between about 20 mm and about 50 mm in diameter.

47. The apparatus according to claim 45, wherein said column has dimensions of between about 50 cm and about 150 cm in length and between about 5cm and about 25 cm in diameter.

48. The apparatus according to claim 44, wherein said separation media is packed into a capillary tube.

49. The apparatus according to claim 48, wherein said tube is about 50  $\mu\text{m}$  in diameter.

50. The apparatus according to claim 44, wherein said separation media is layered onto a plate.

51. The apparatus according to claim 50, wherein said plate is between about 100  $\mu\text{m}$  and about 300  $\mu\text{m}$  in thickness.

52. The apparatus according to claim 45, wherein the packing is between atmospheric pressure and about 7,000 psi.



- 63 -

53. The apparatus according to claim 44, wherein said separation media is in a simulated moving bed.

54. The apparatus according to claim 44, wherein said separation media is in a fluidized bed.

55. An apparatus for contacting a sample with separation media comprising crosslinked protein crystals, said apparatus being selected from the group consisting of columns, membranes, plates, batches and capillary tubes.

56. The apparatus according to claim 55, wherein said apparatus is a column and said separation media is packed into said column.

57. The apparatus according to claim 56, wherein said column has dimensions of between about 5 cm and about 25 cm in length and between about 20 mm and about 50 mm in diameter.

58. The apparatus according to claim 56, wherein said column has dimensions of between about 50 cm and about 150 cm in length and between about 5 cm and about 25 cm in diameter.

59. The apparatus according to claim 55, wherein said apparatus is a capillary tube and said separation media is packed into said capillary tube.

- 64 -

60. The apparatus according to claim 59, wherein said tube is about 50  $\mu\text{m}$  in diameter.

61. The apparatus according to claim 55, wherein said apparatus is a plate and said separation media is layered onto said plate.

62. The apparatus according to claim 61, wherein said plate is between about 100  $\mu\text{m}$  and about 300  $\mu\text{m}$  in thickness.

63. The apparatus according to claim 55, wherein said apparatus is a membrane and said separation media is layered onto said membrane.

64. The apparatus according to claim 63, wherein said membrane is between about 100  $\mu\text{m}$  and 500  $\mu\text{m}$  in thickness.

1 / 46

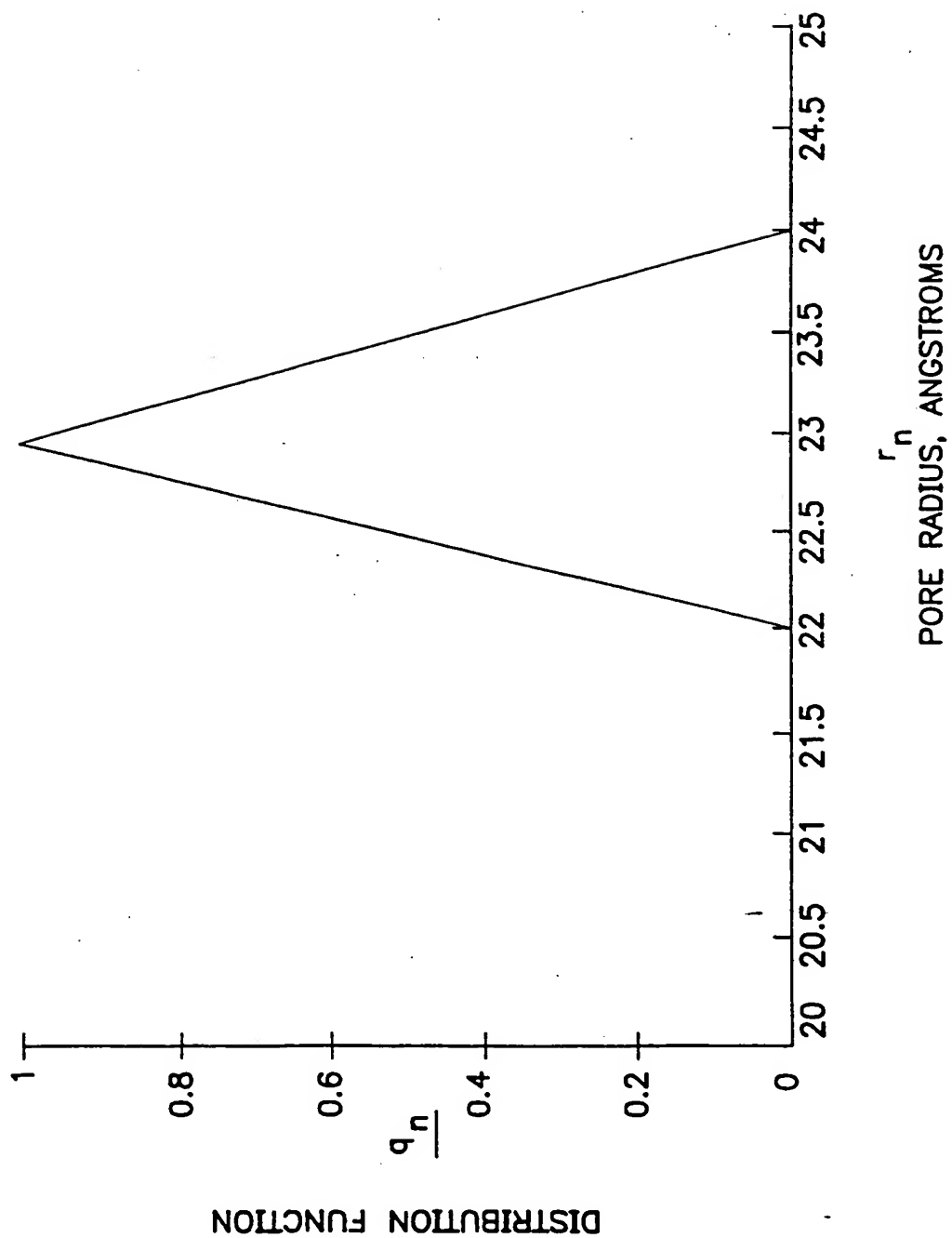


FIG. 1

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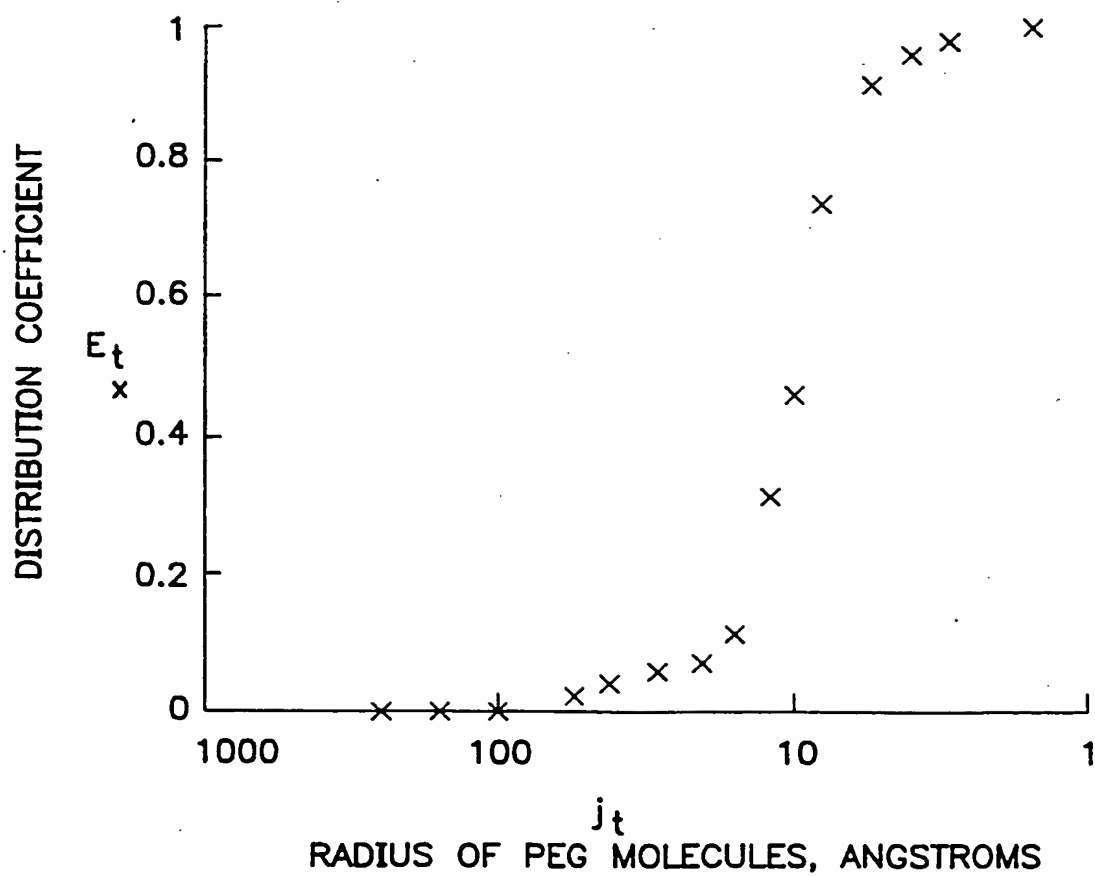


FIG. 2

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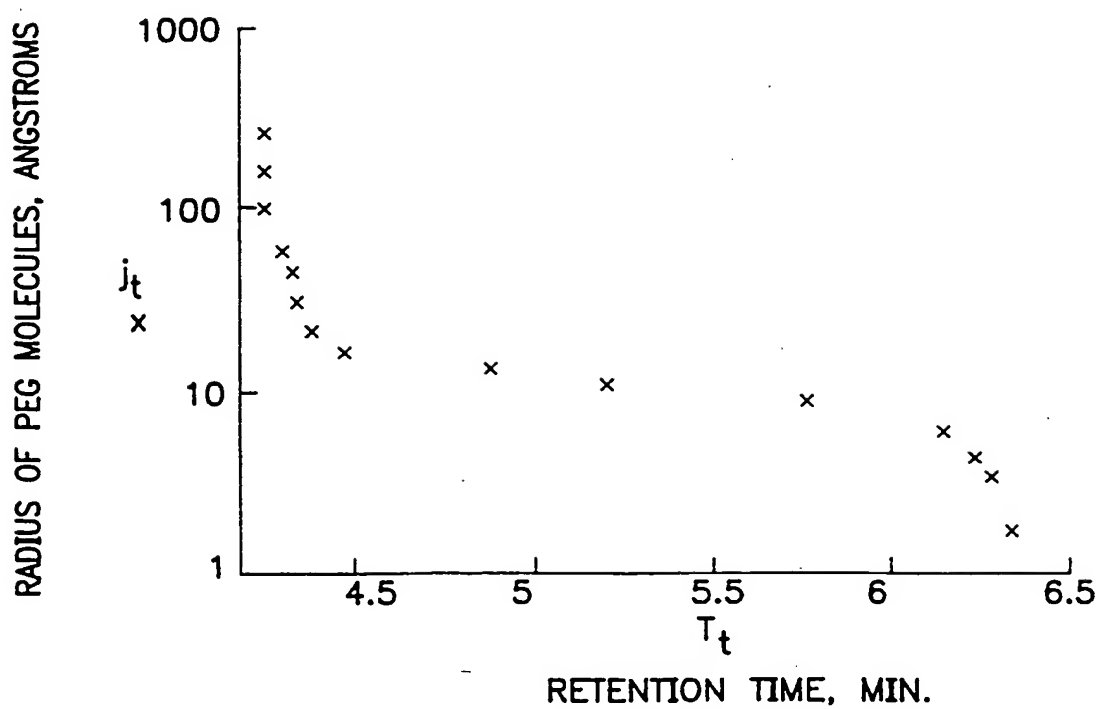


FIG. 3

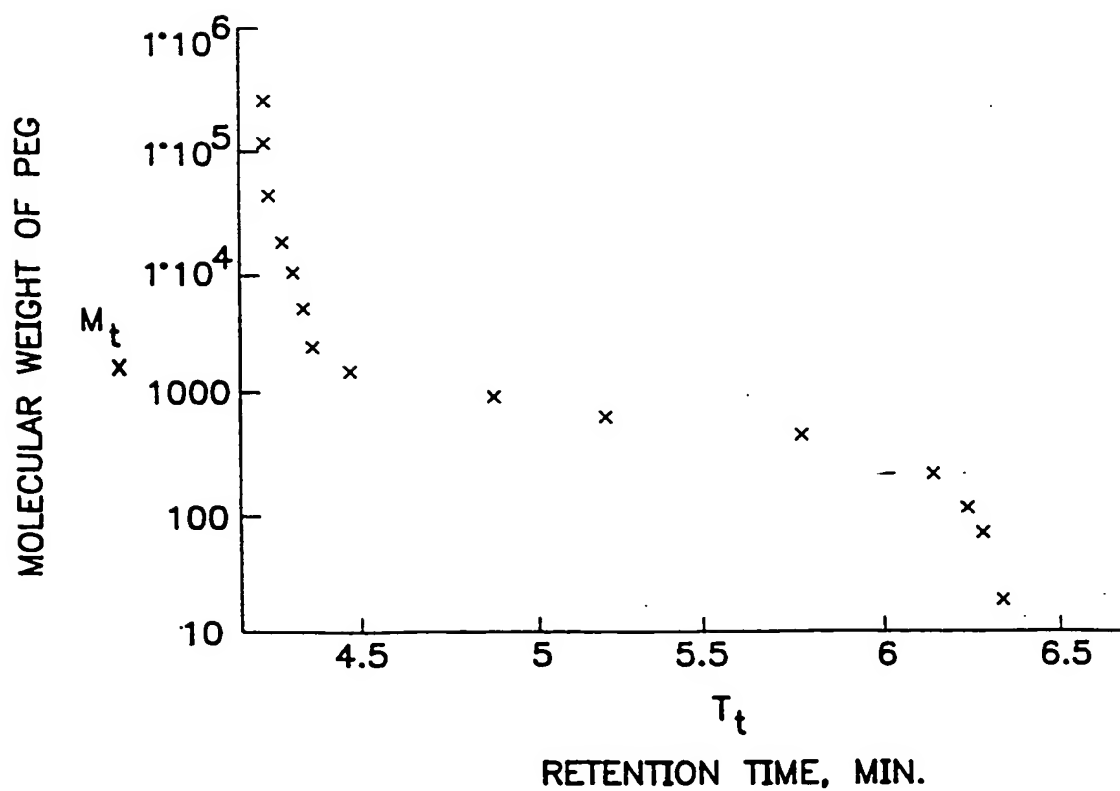


FIG. 4

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4/46

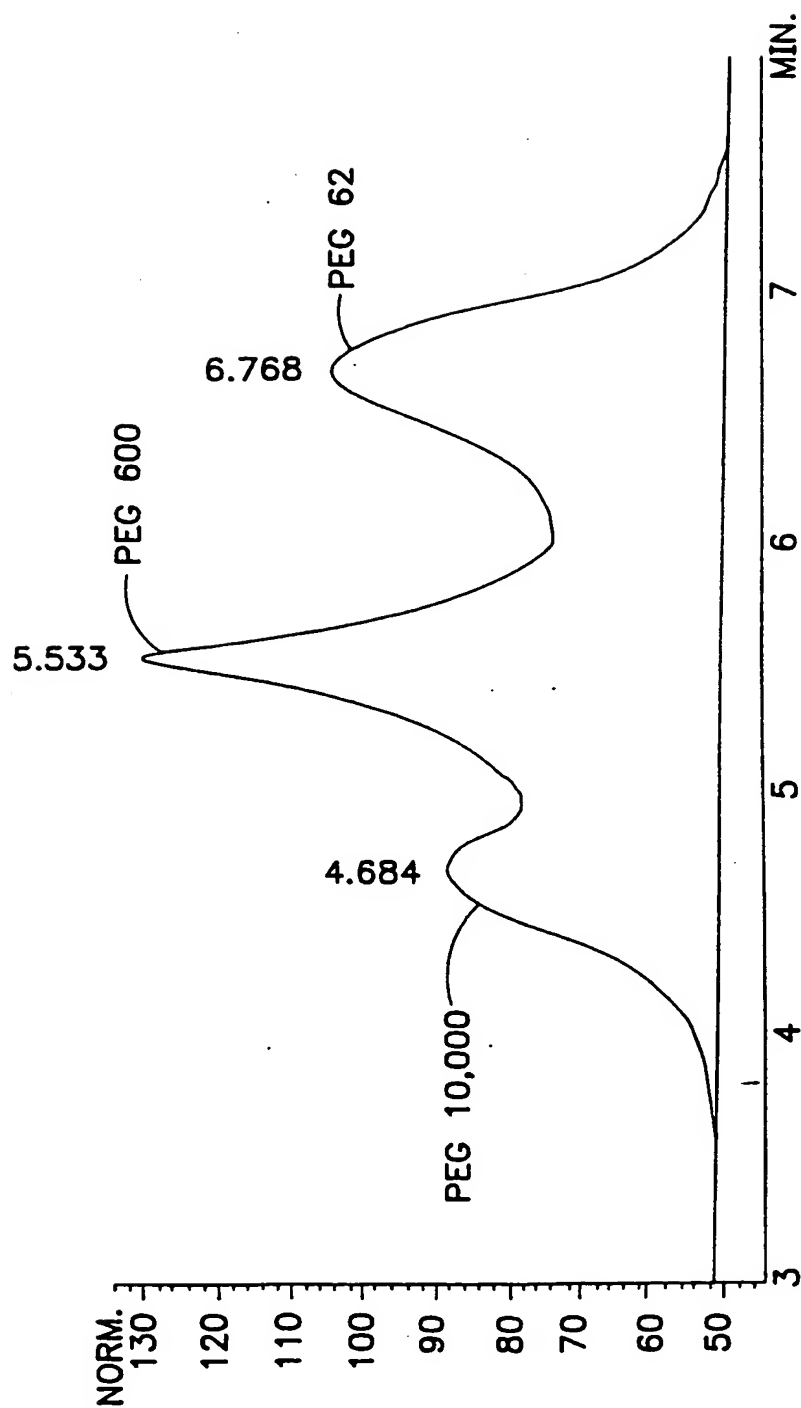


FIG. 5

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5/46

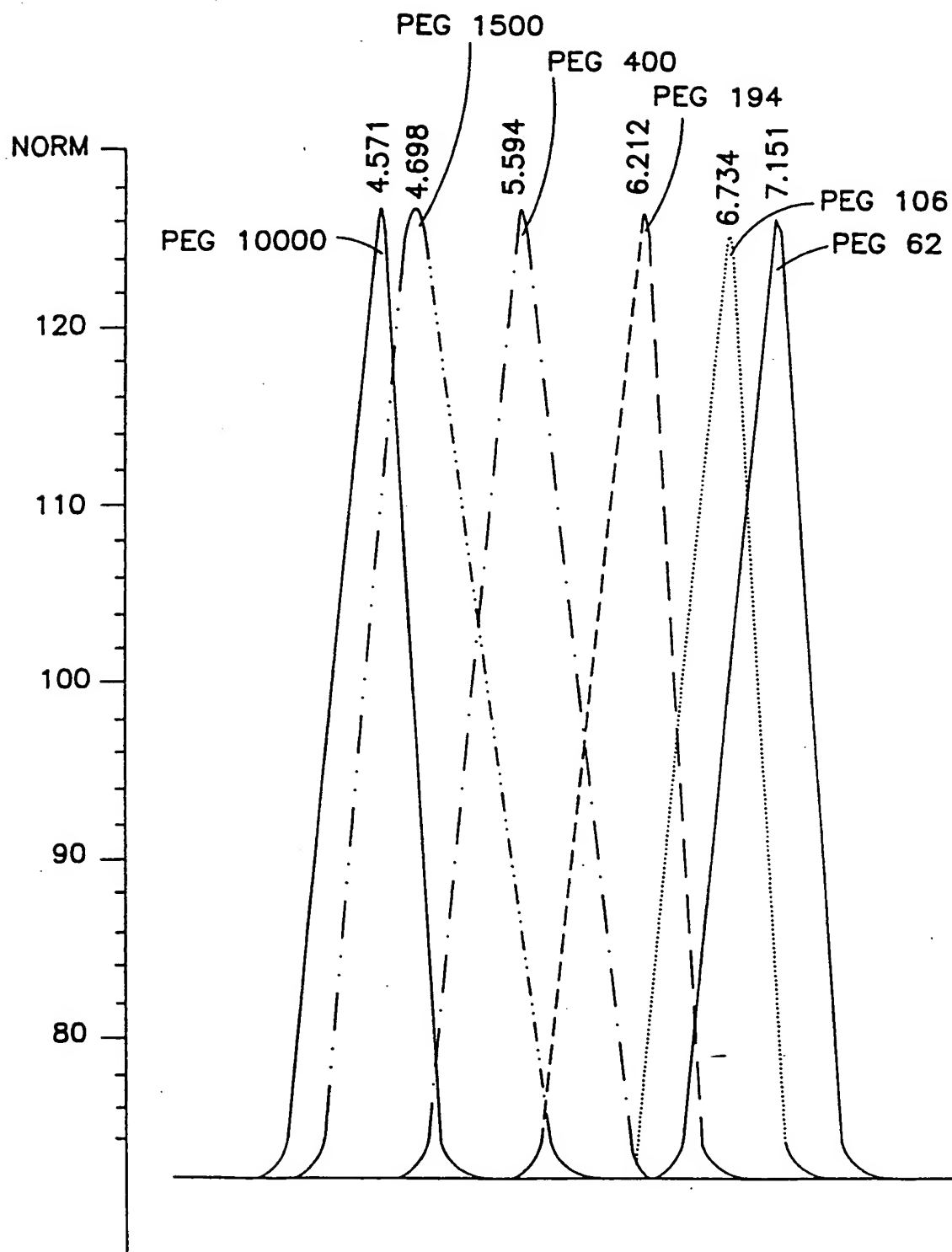


FIG. 6

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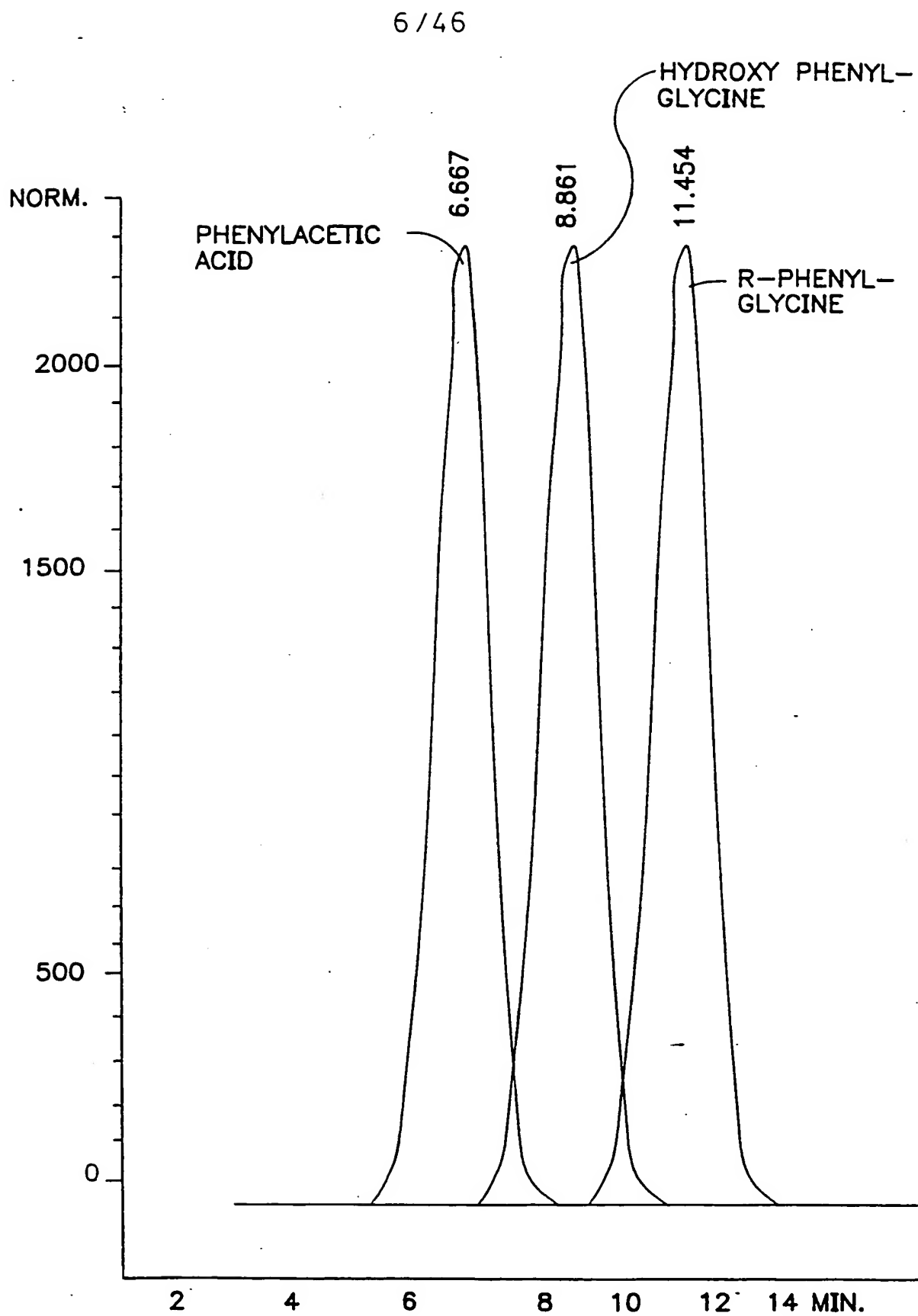


FIG. 7

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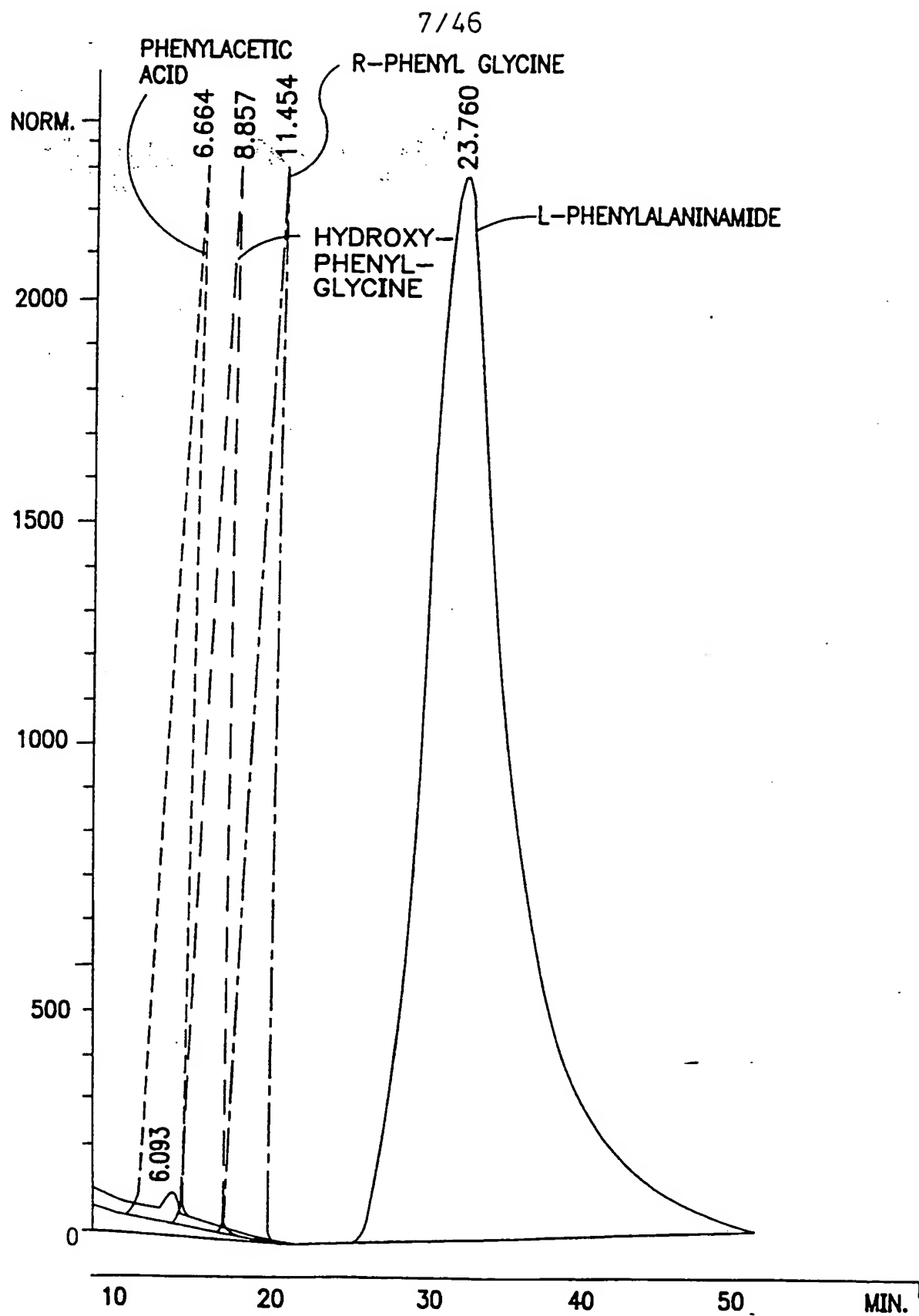


FIG. 8

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8/46

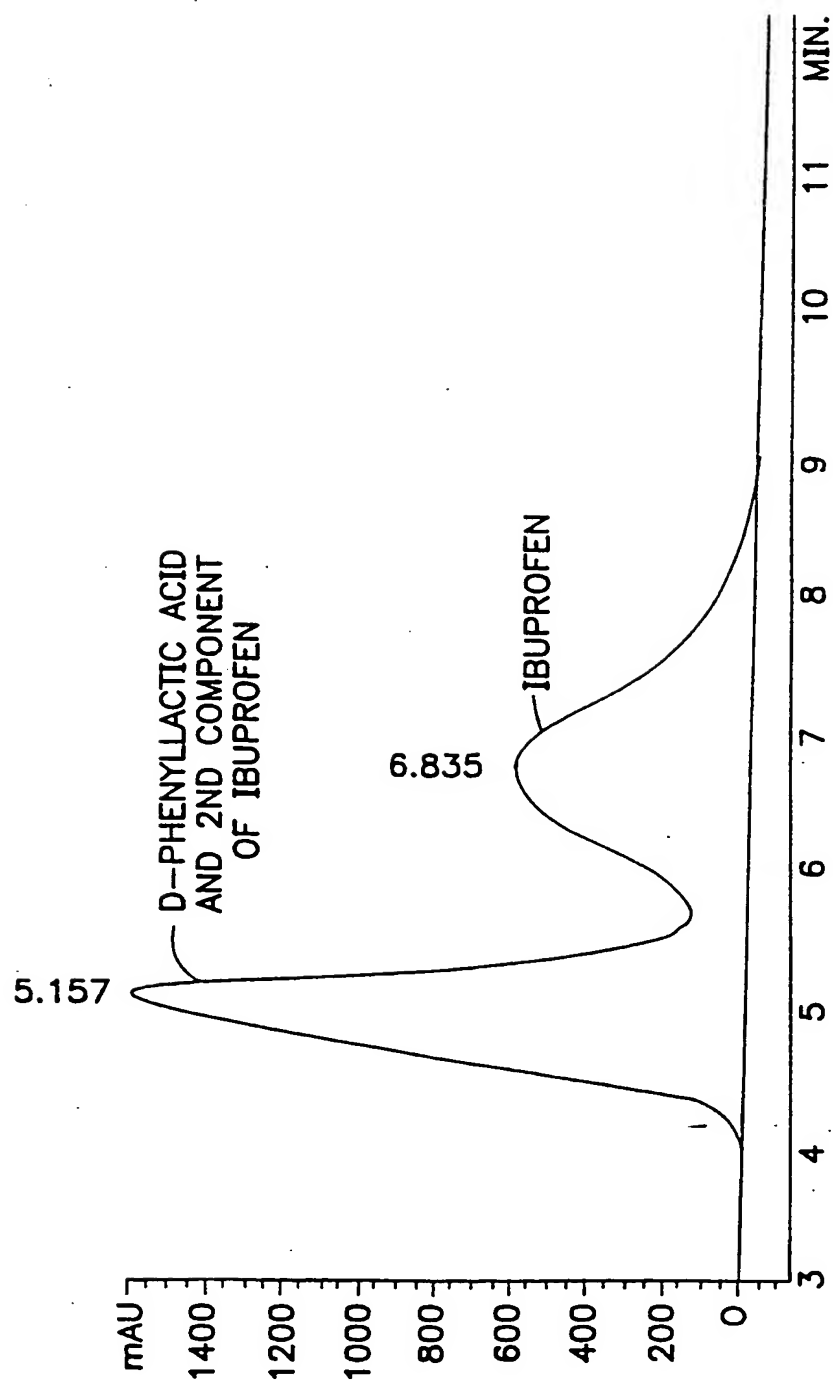


FIG. 9

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9/46

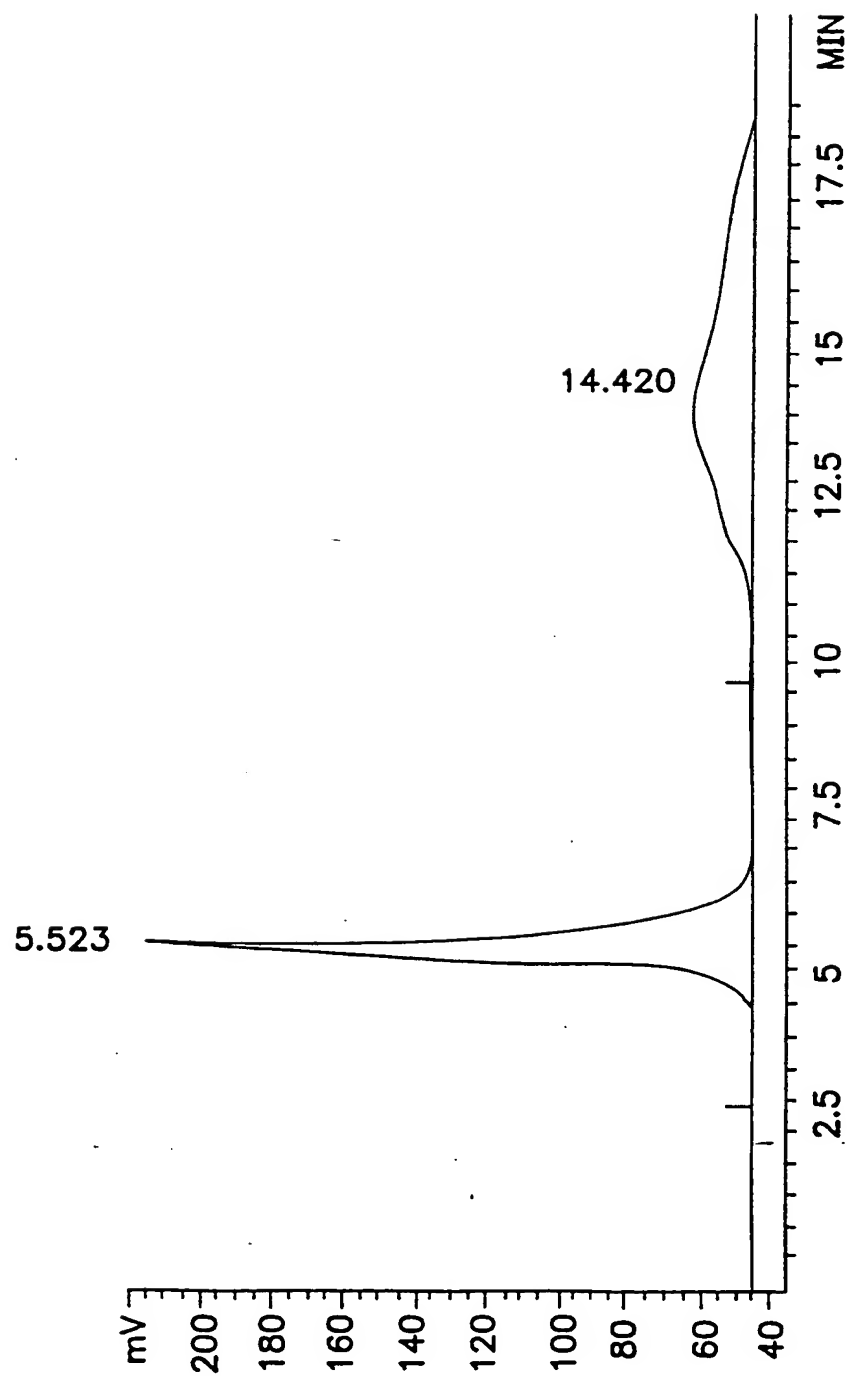


FIG. 10

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10/46

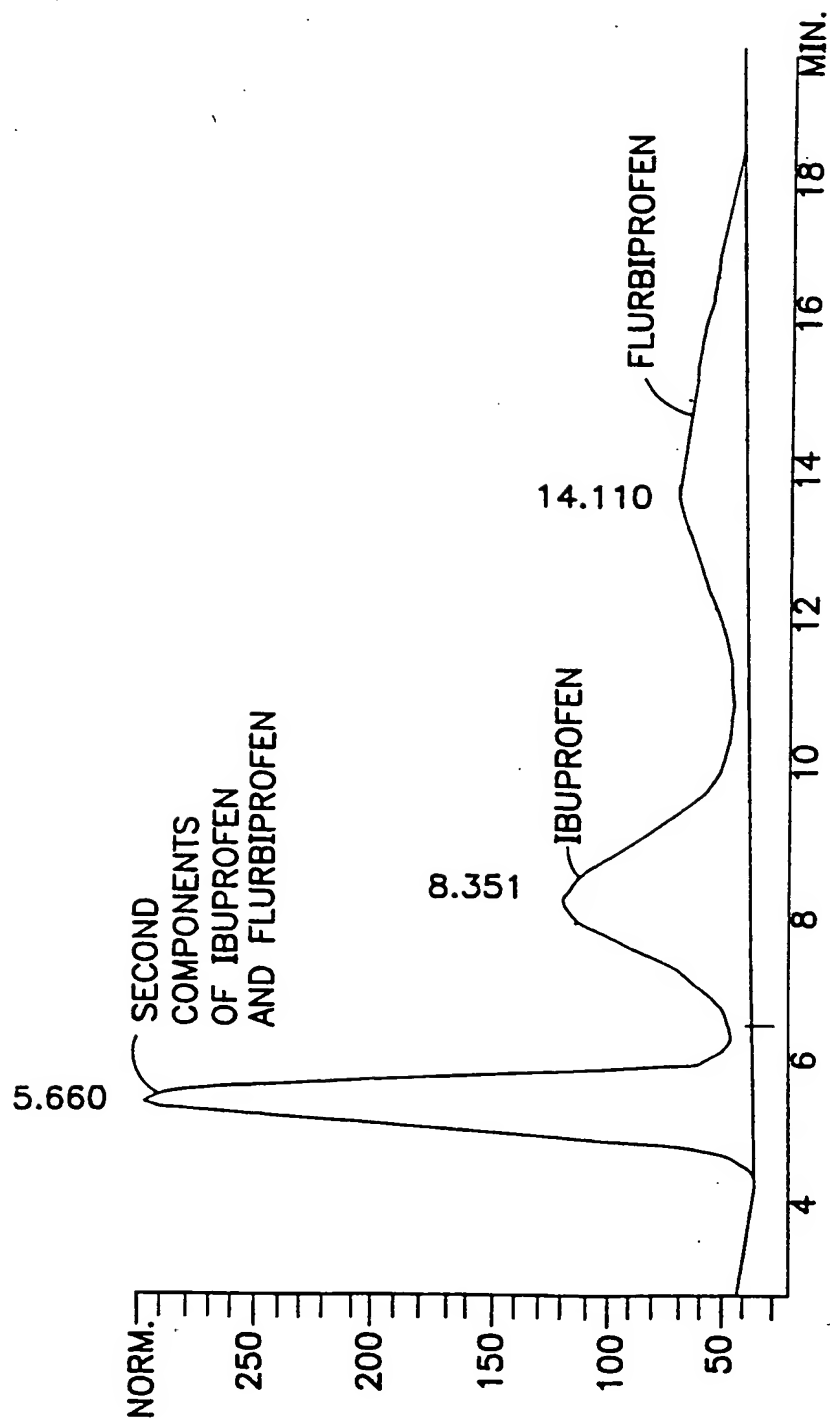


FIG. 11

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11/46

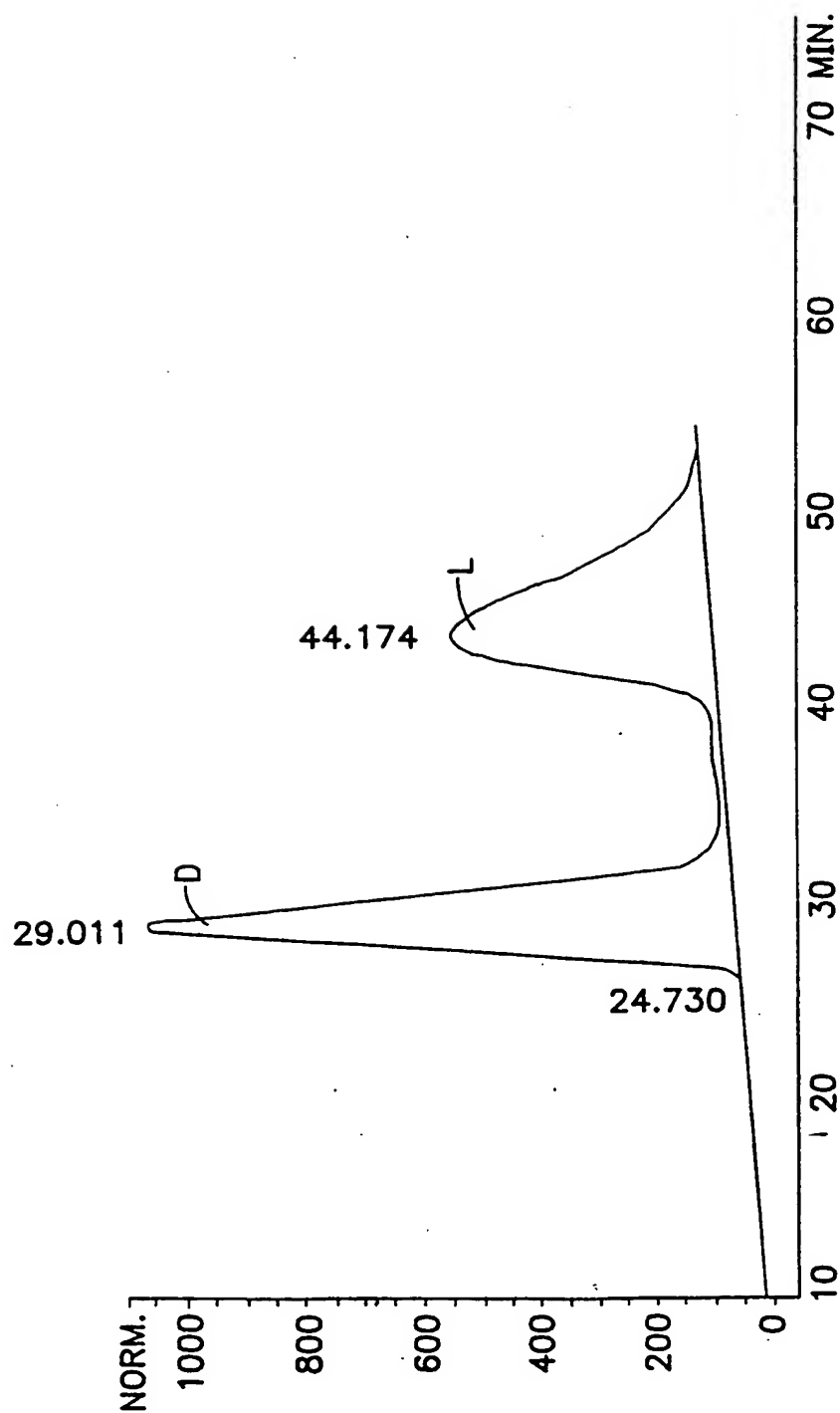


FIG. 12

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12 / 46

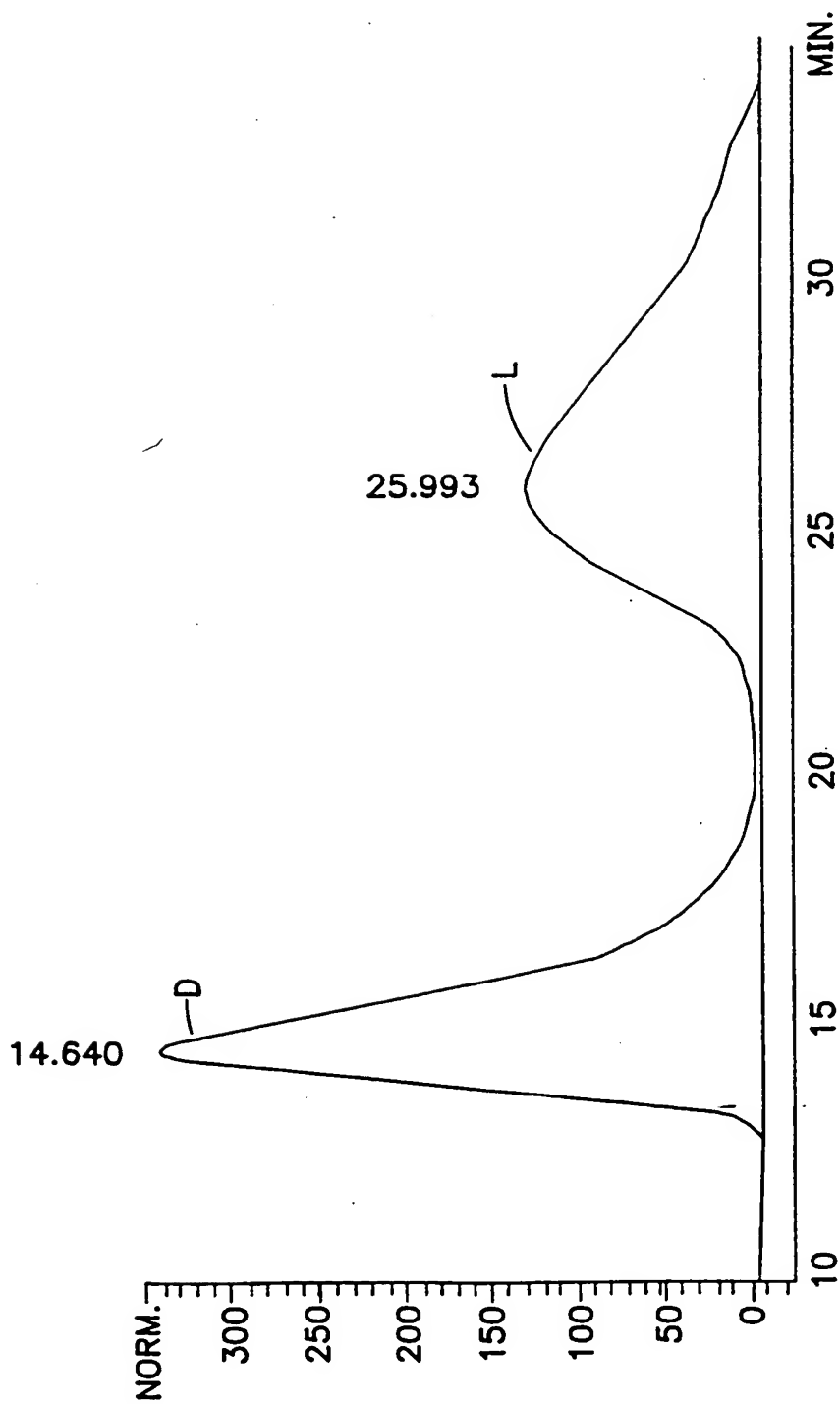


FIG. 13

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13/46

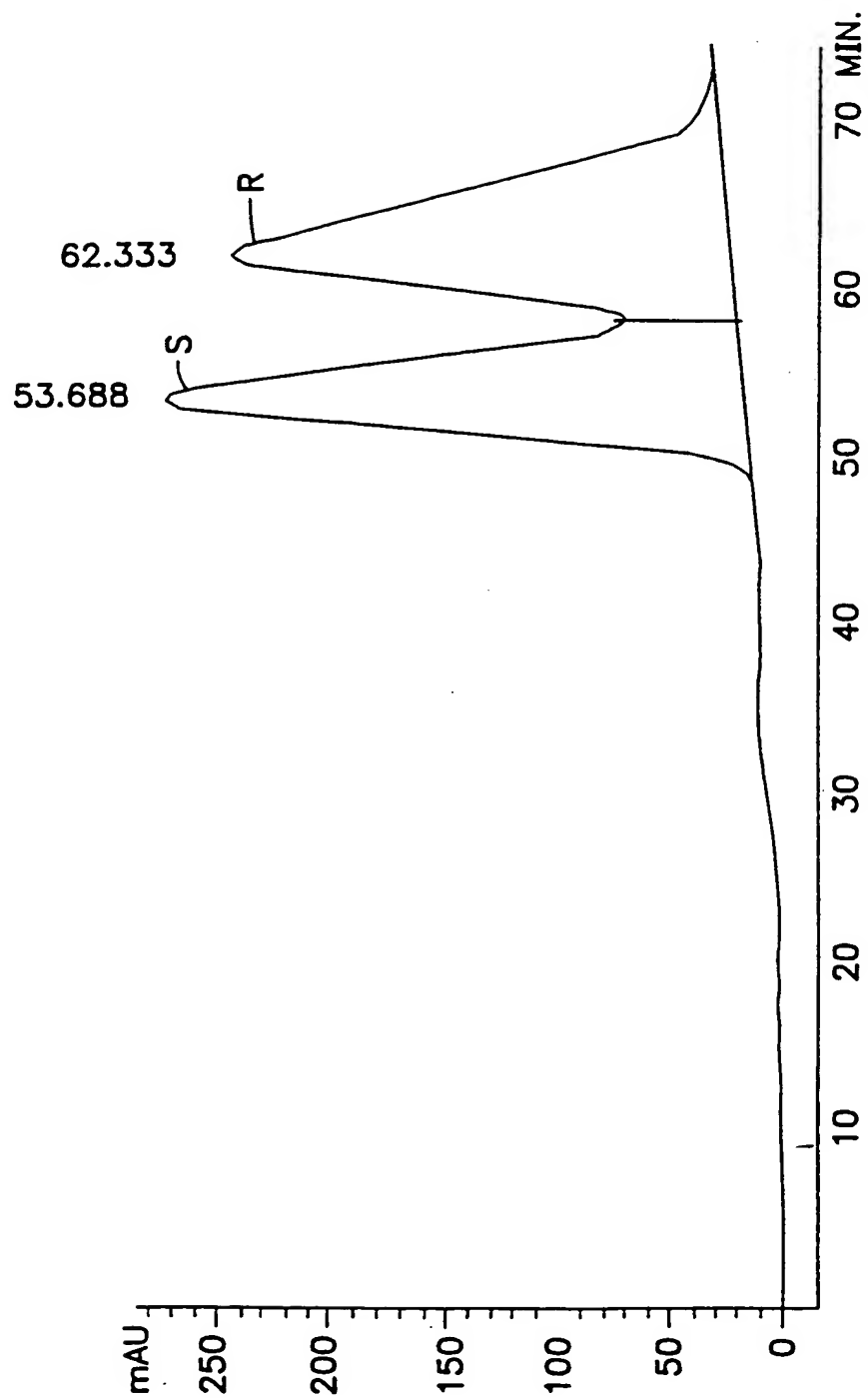


FIG. 14

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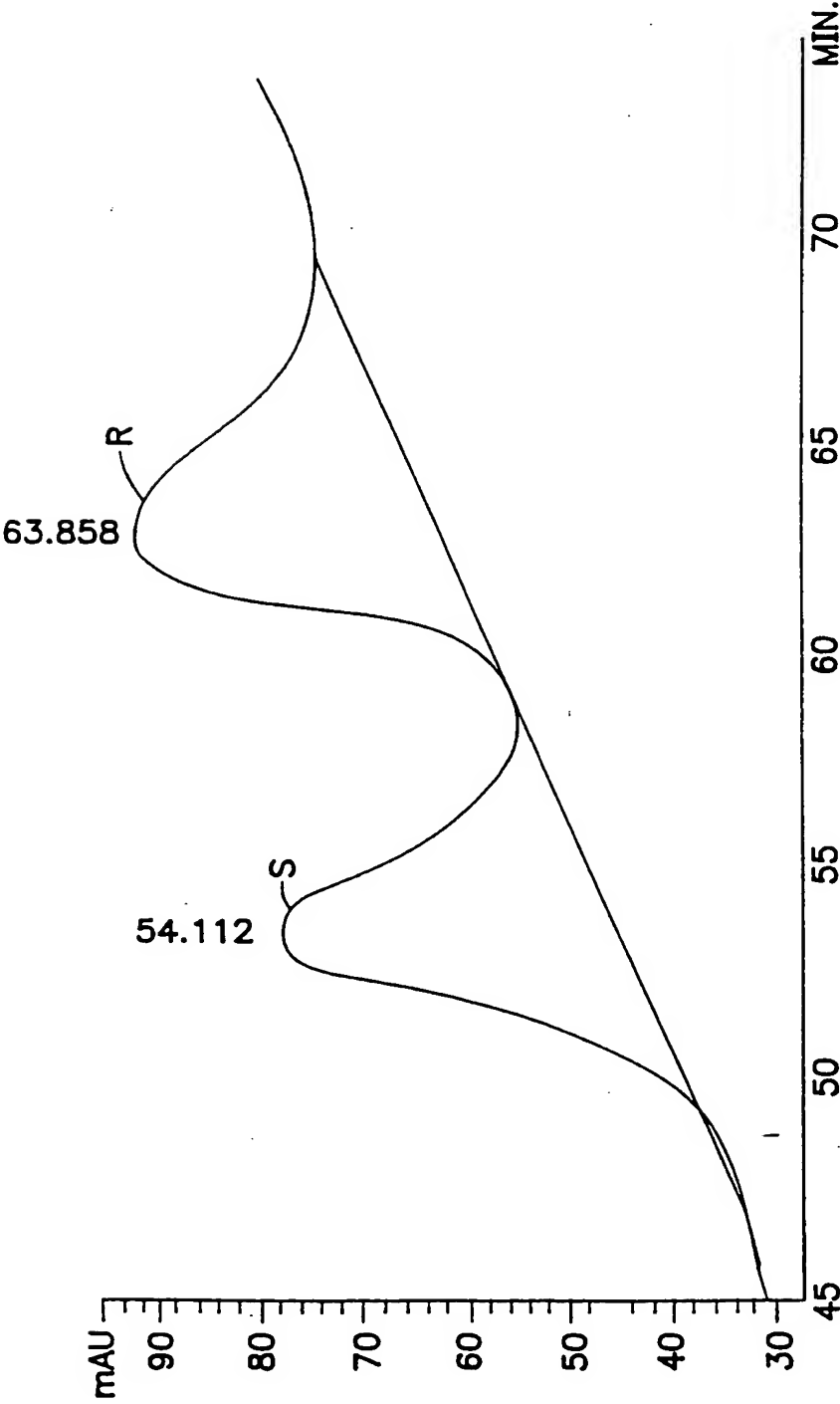


FIG. 15

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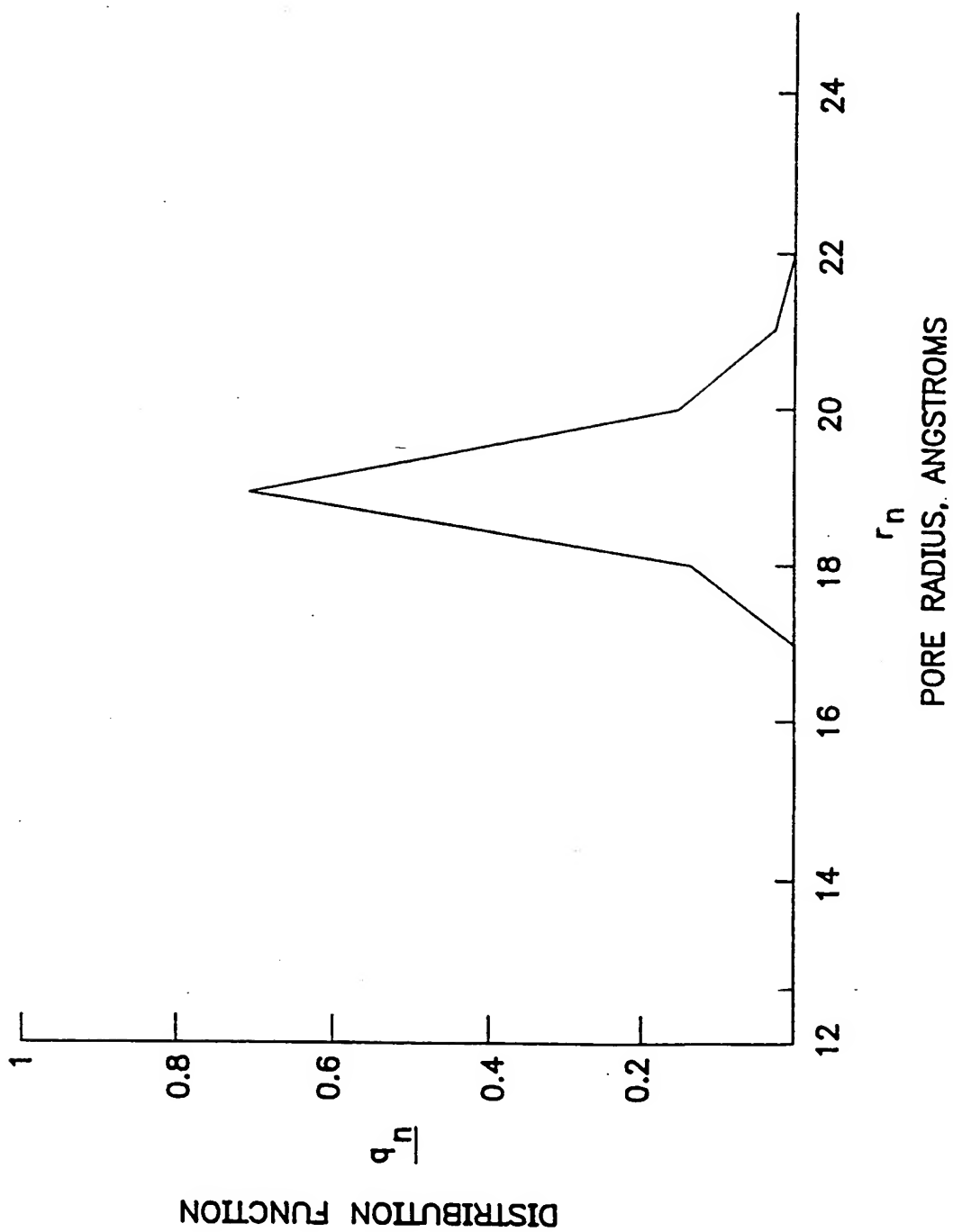


FIG. 16

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16/46

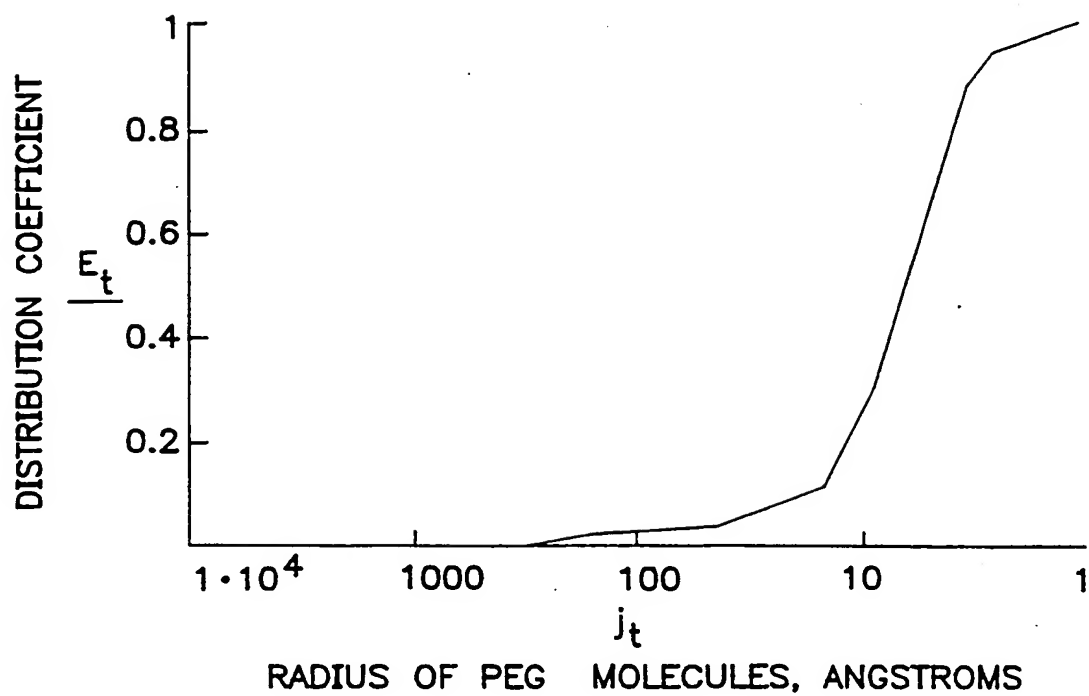


FIG. 17

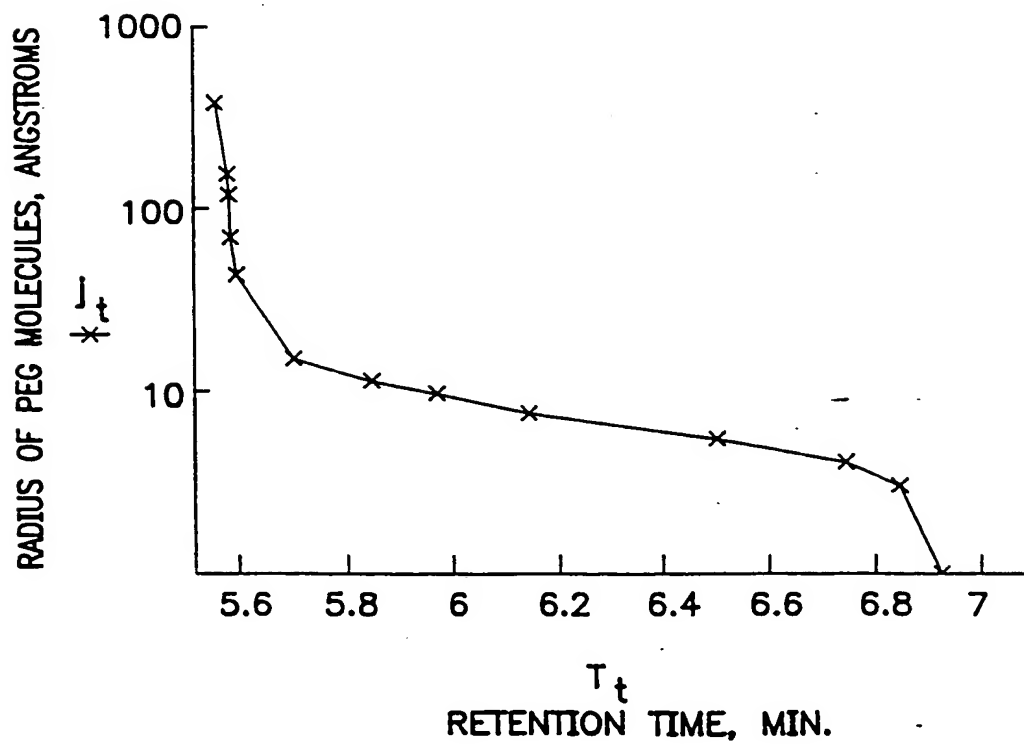


FIG. 18

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17/46

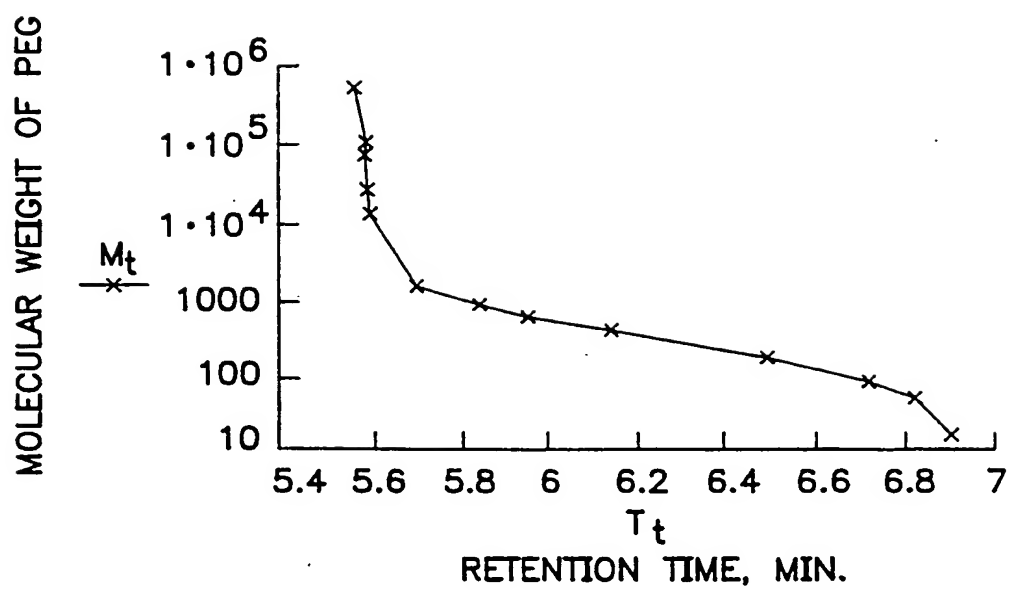


FIG. 19

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18 / 46

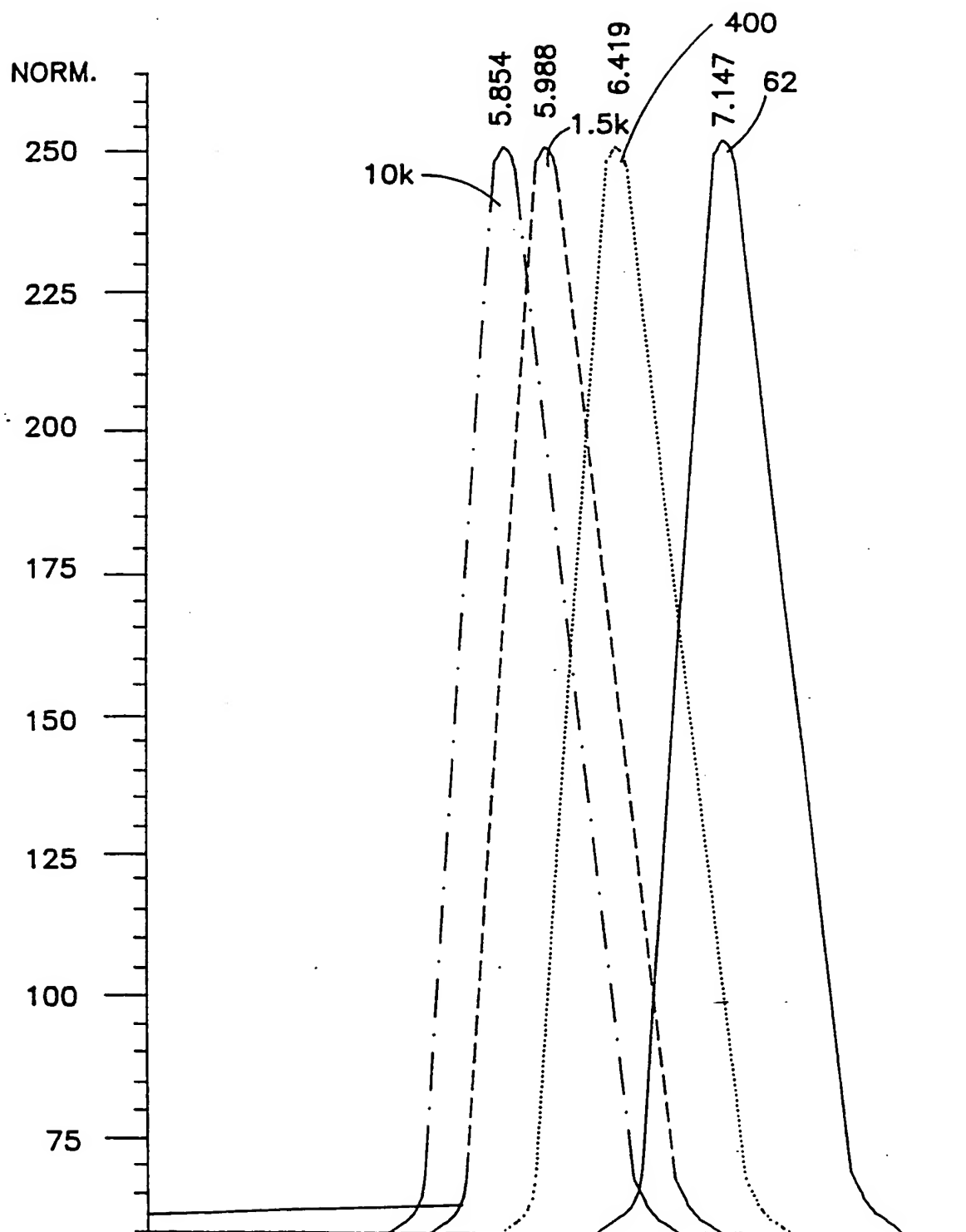


FIG. 20

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19/46

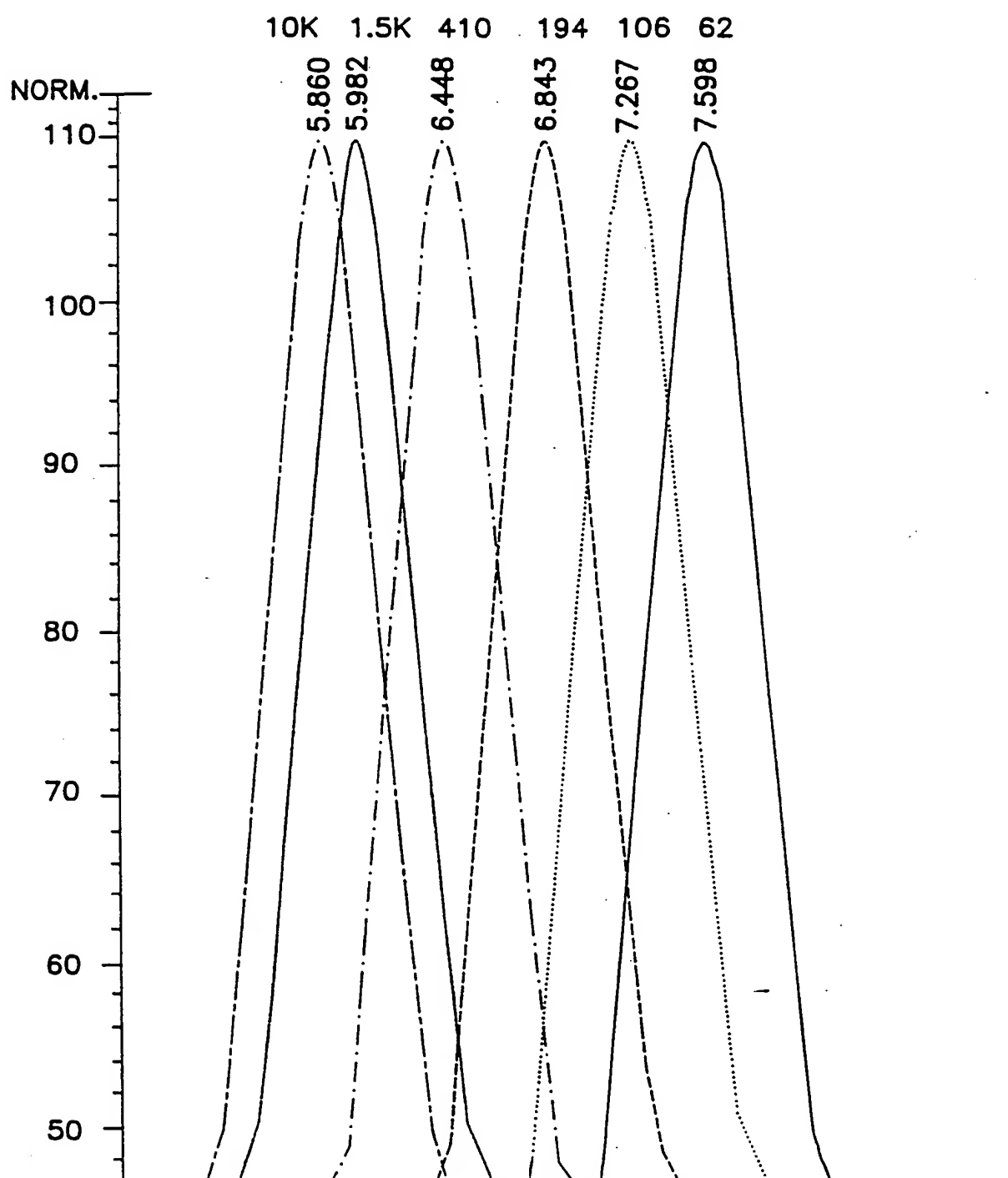


FIG. 21

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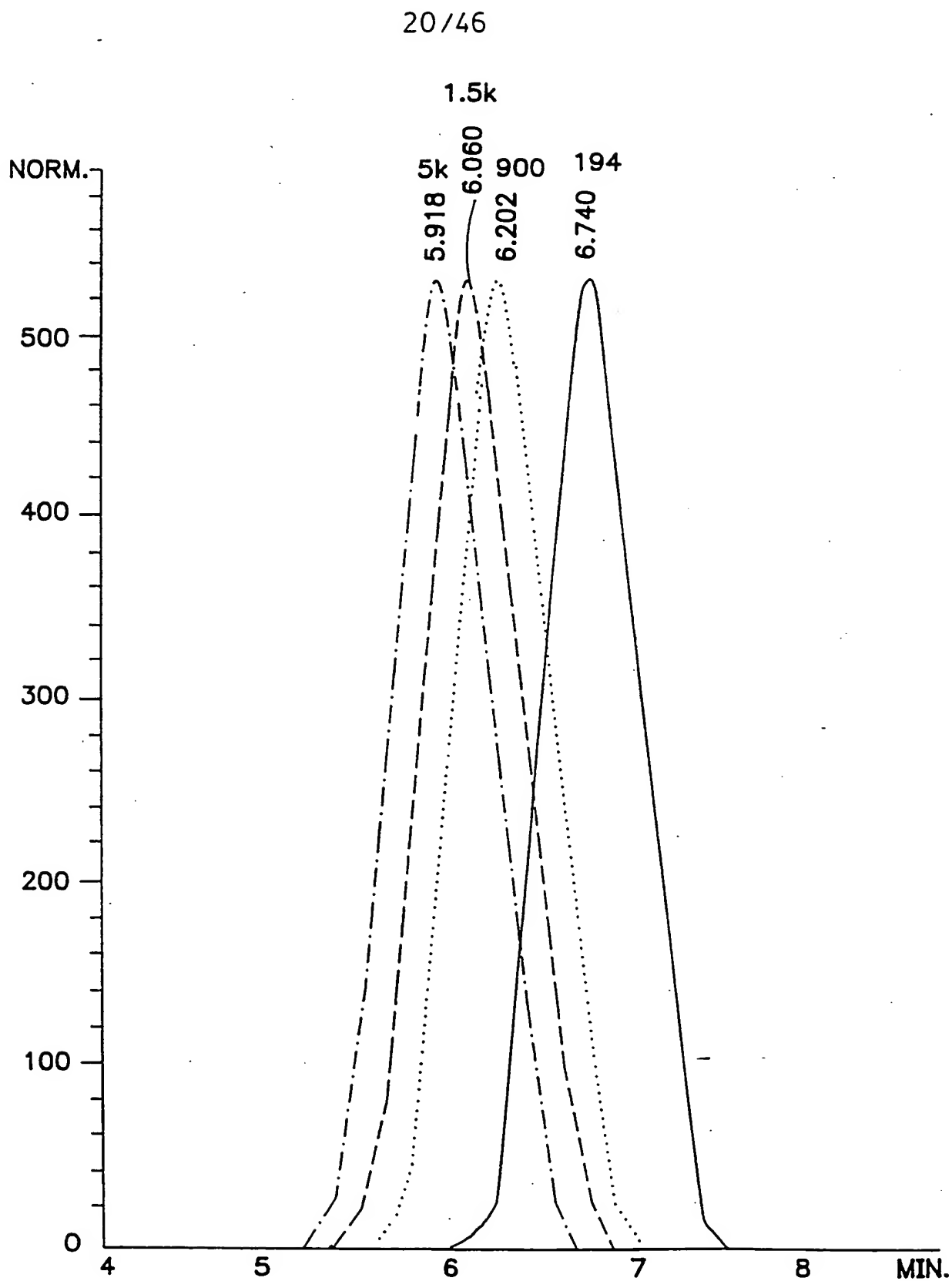


FIG. 22

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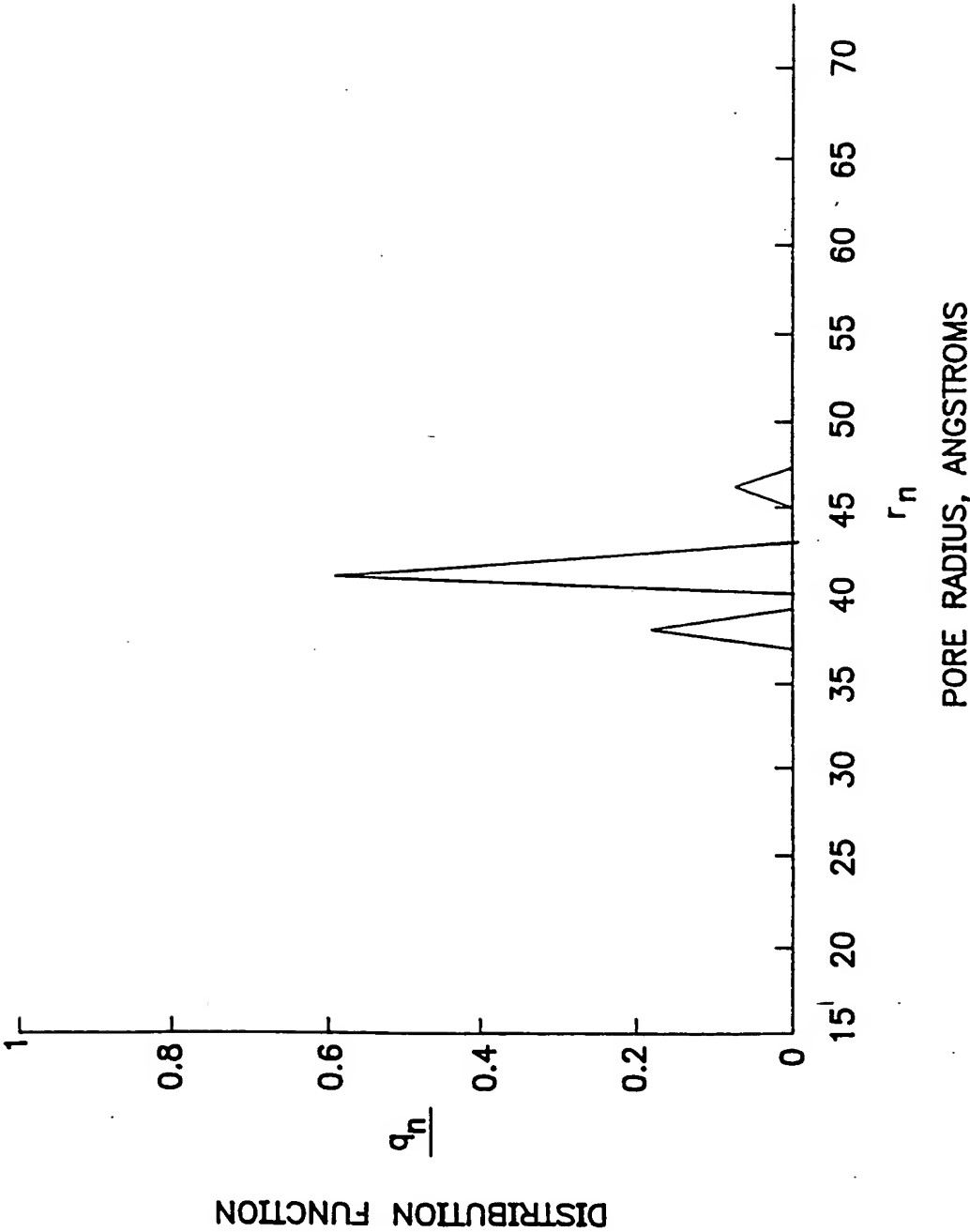


FIG. 23

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22/46

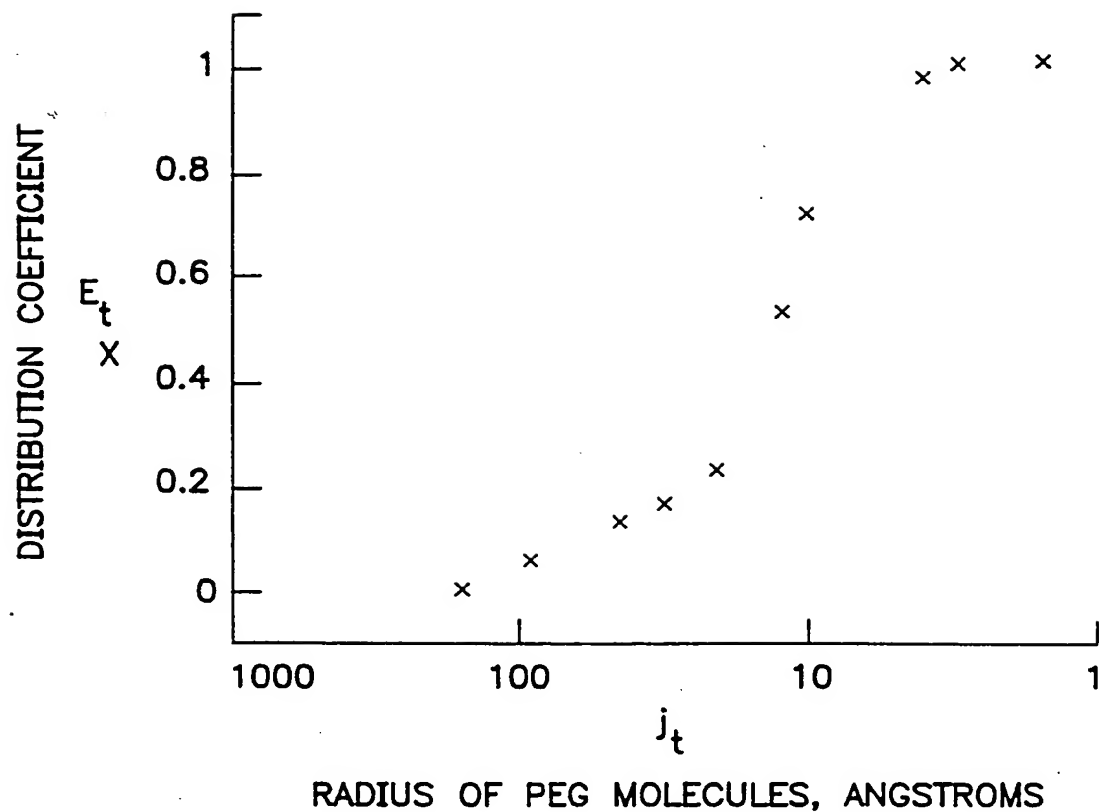


FIG. 24

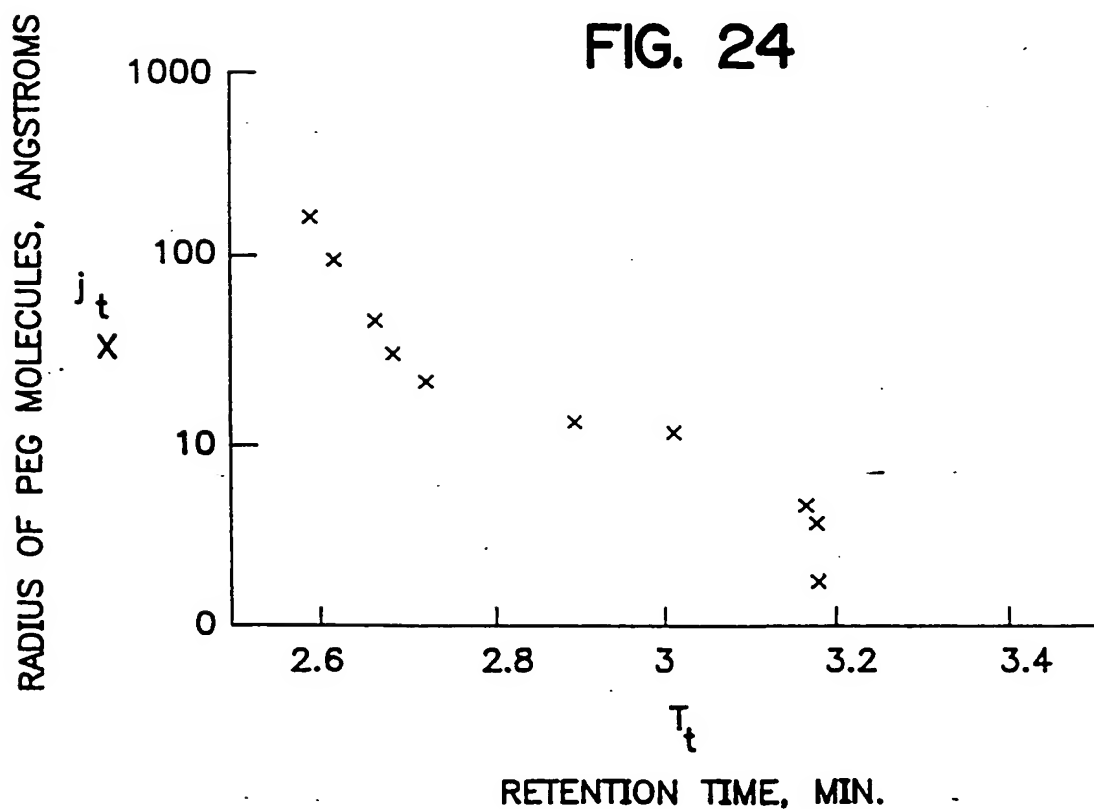


FIG. 25

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23/46

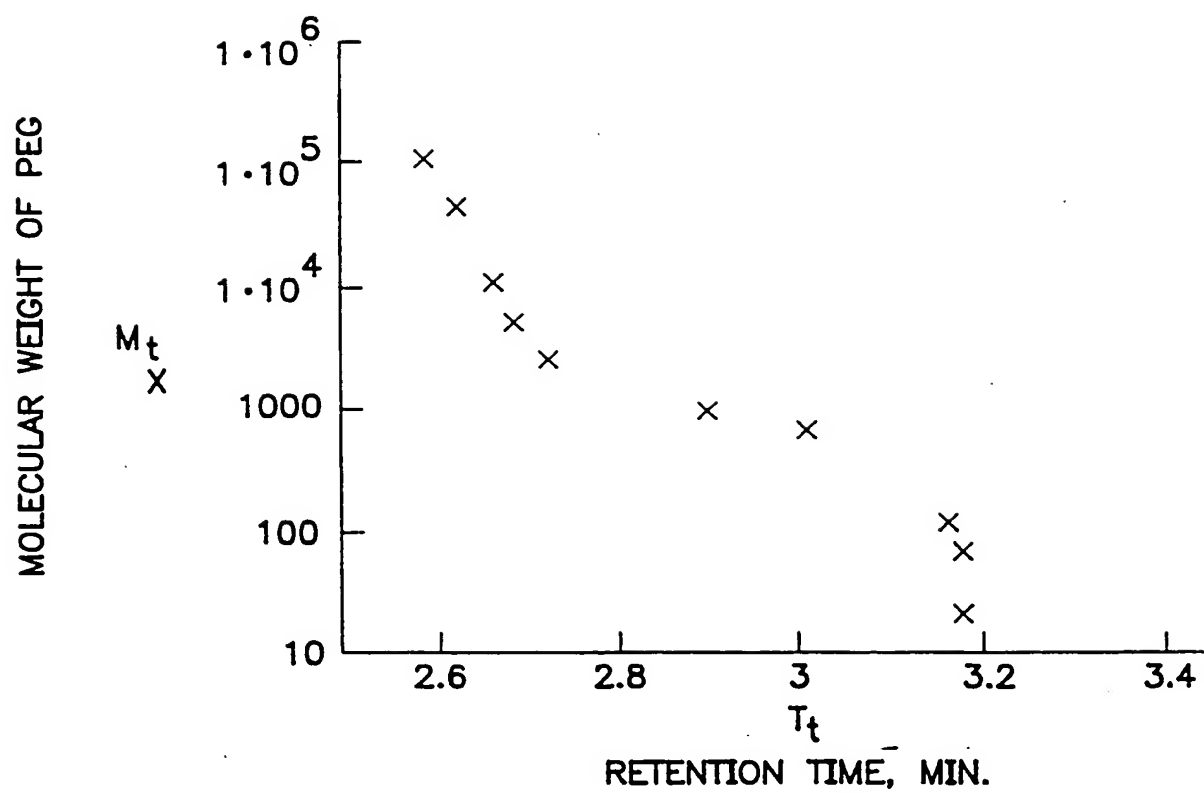


FIG. 26

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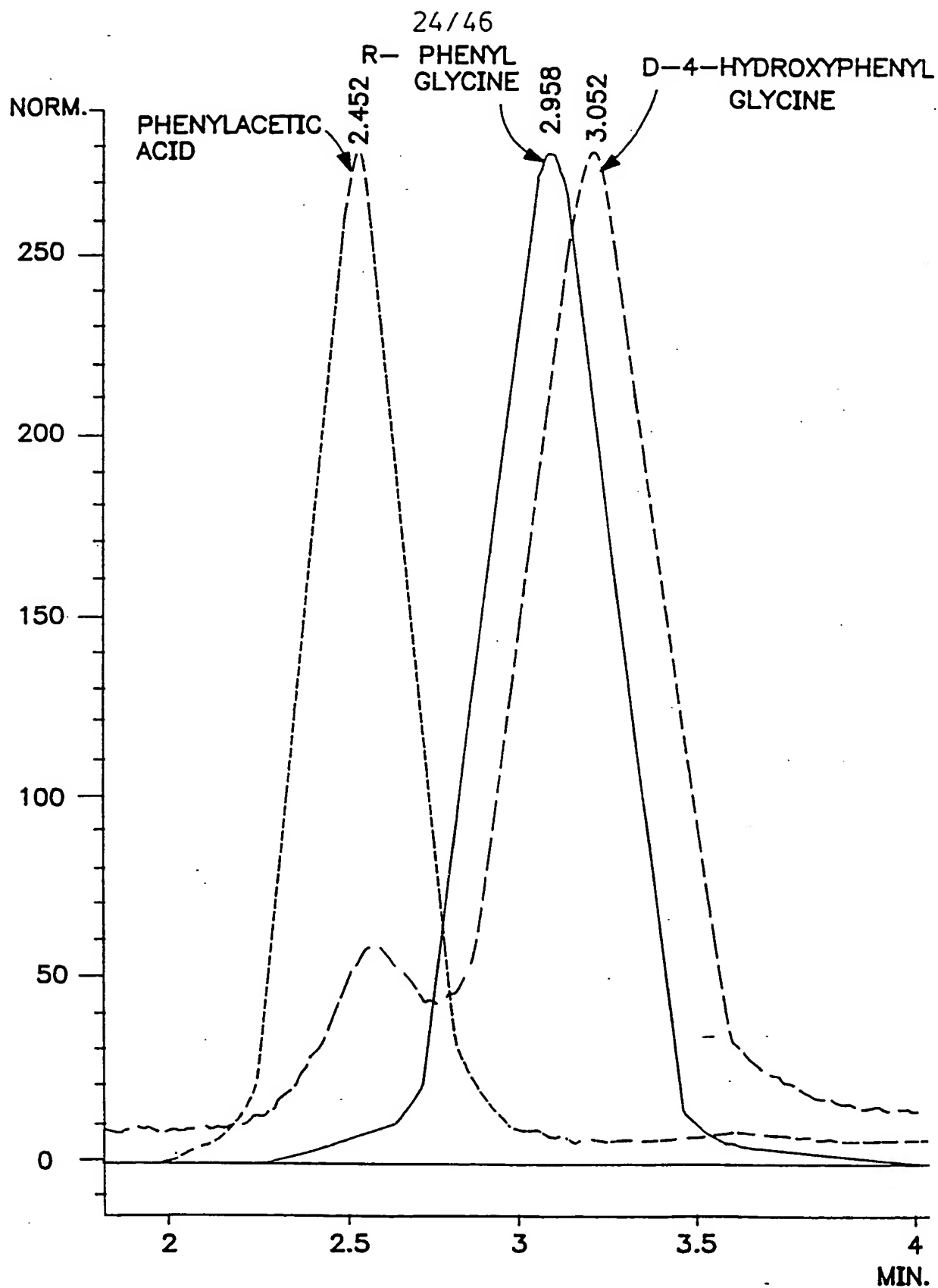
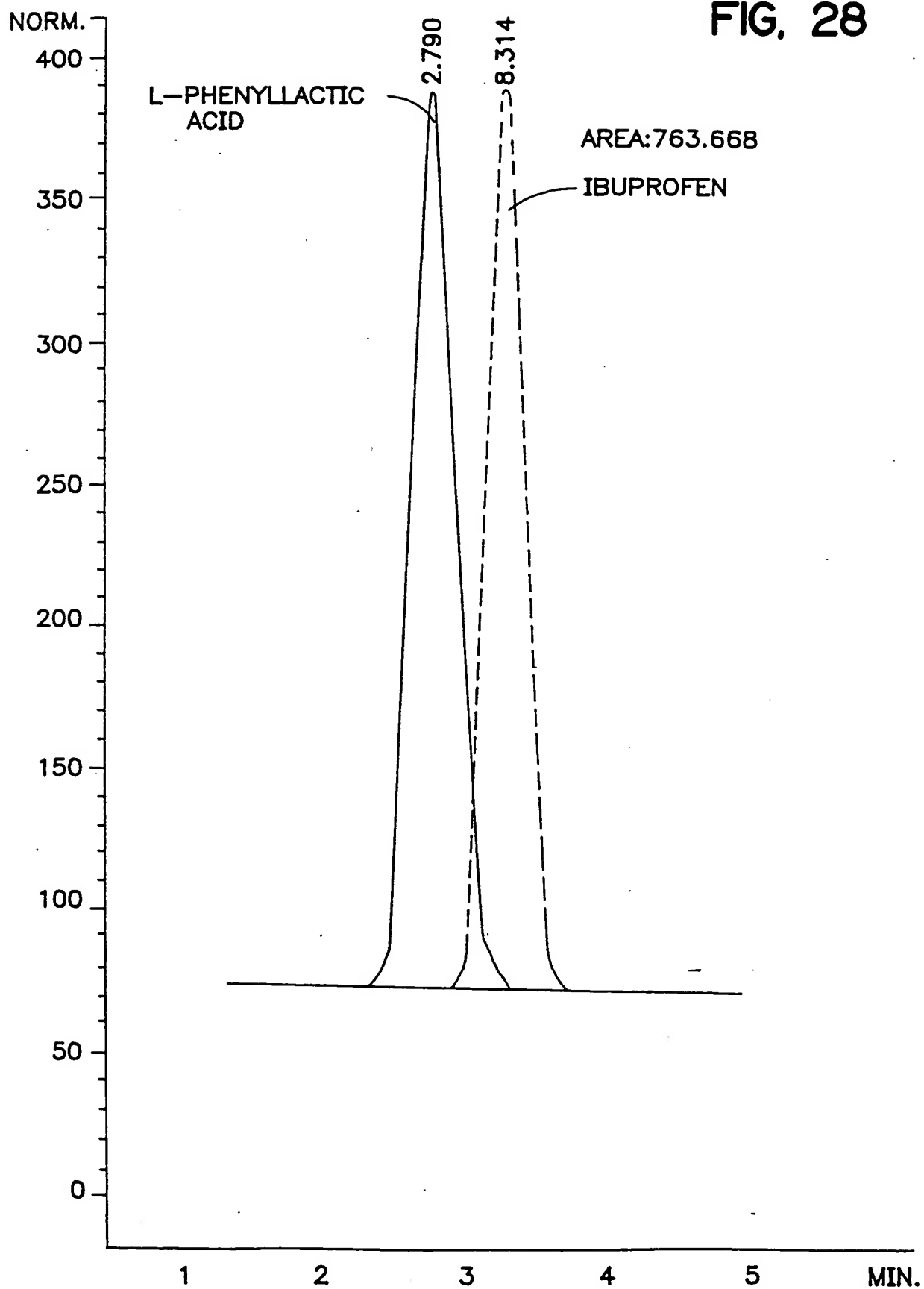


FIG. 27

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25/46

FIG. 28



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26/46

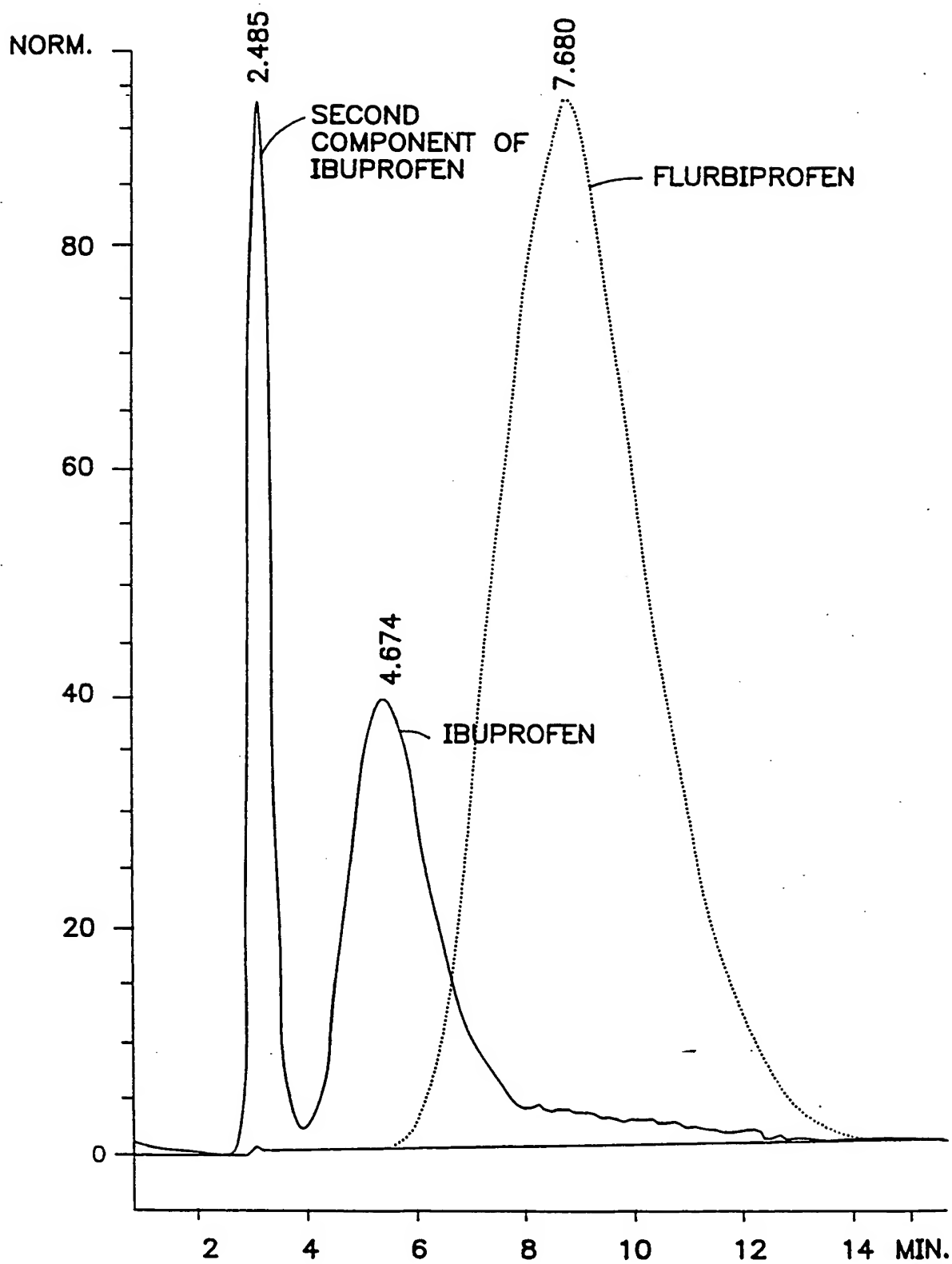
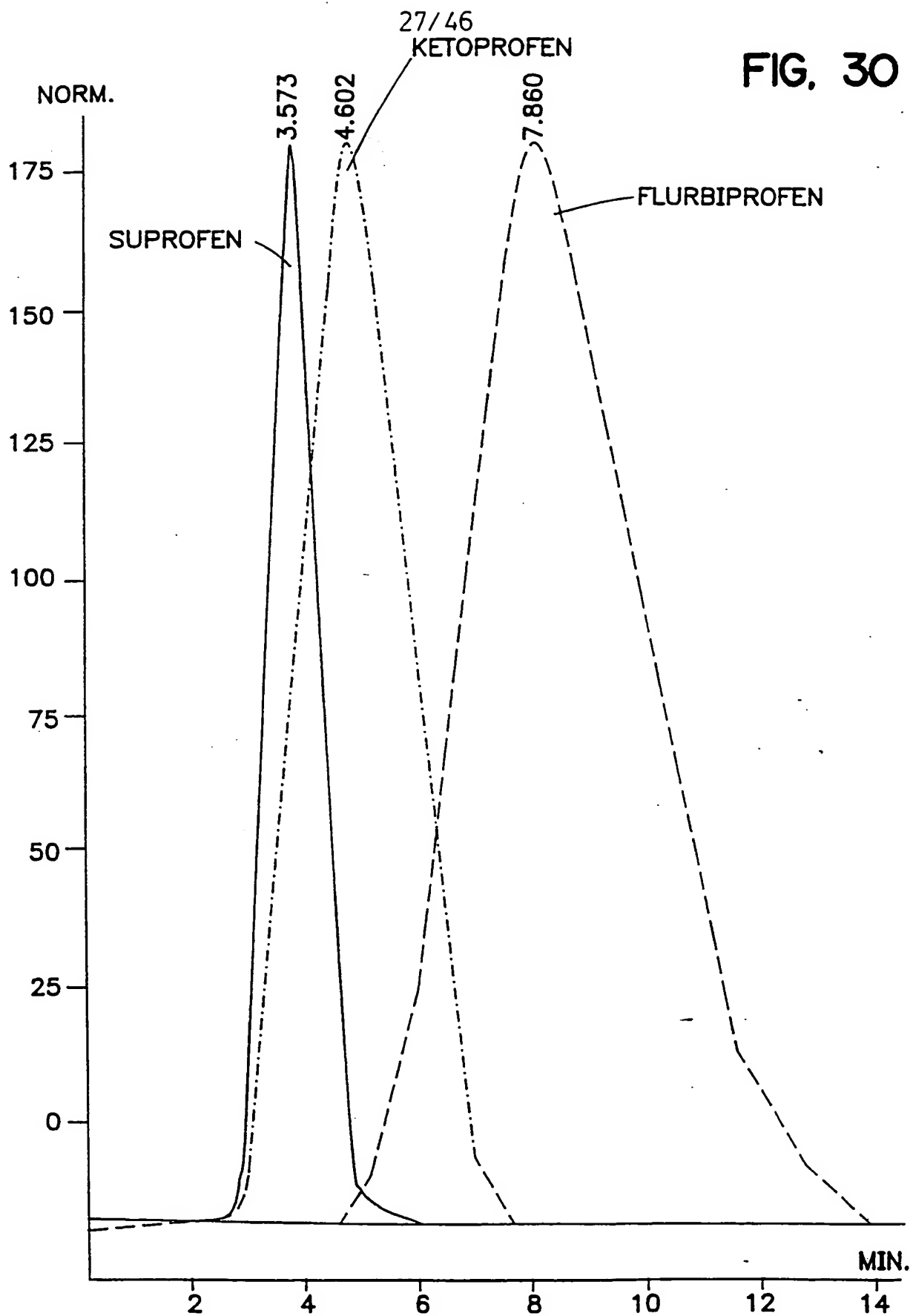


FIG. 29

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FIG. 30



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28/46

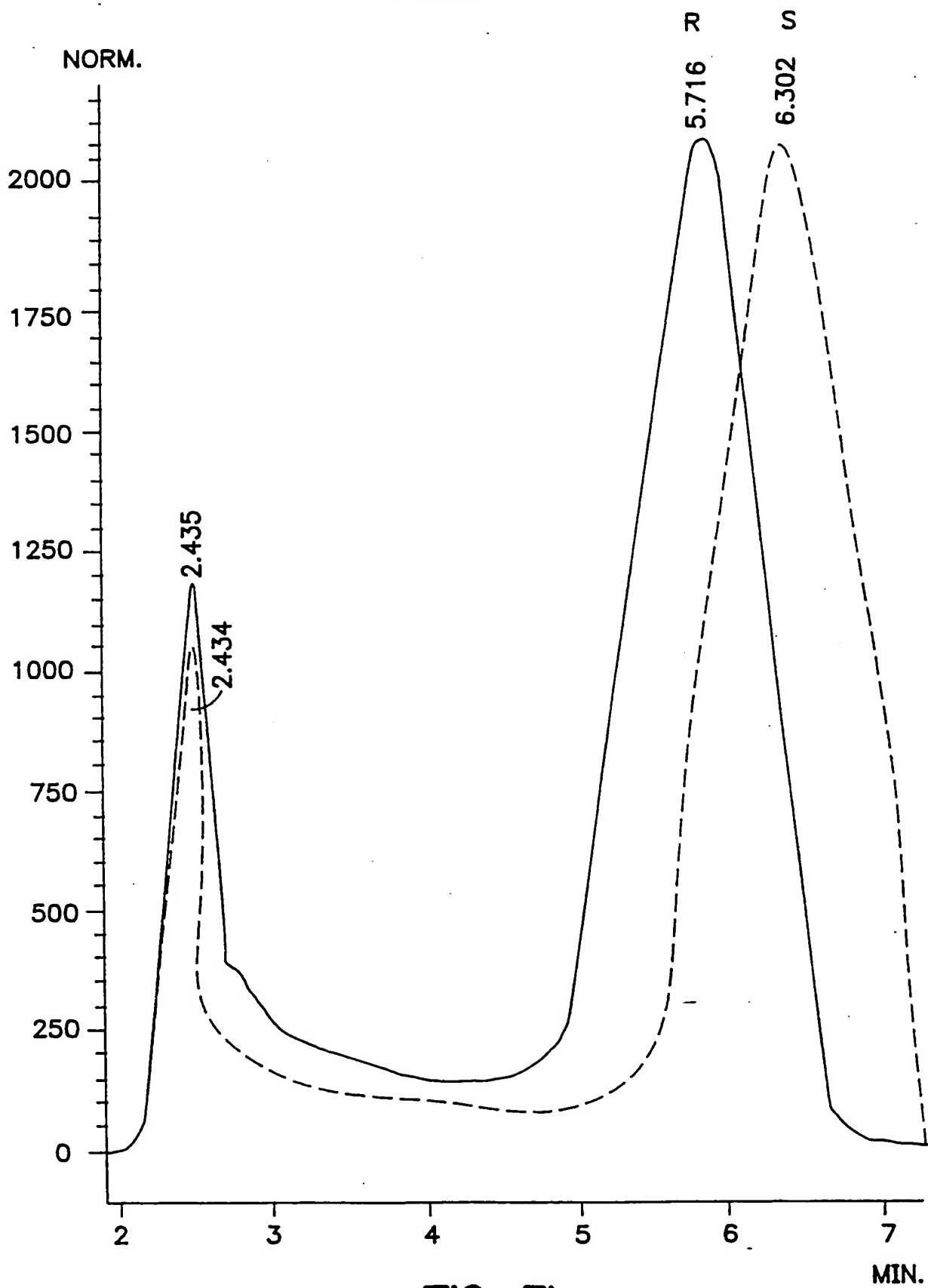


FIG. 31

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29/46

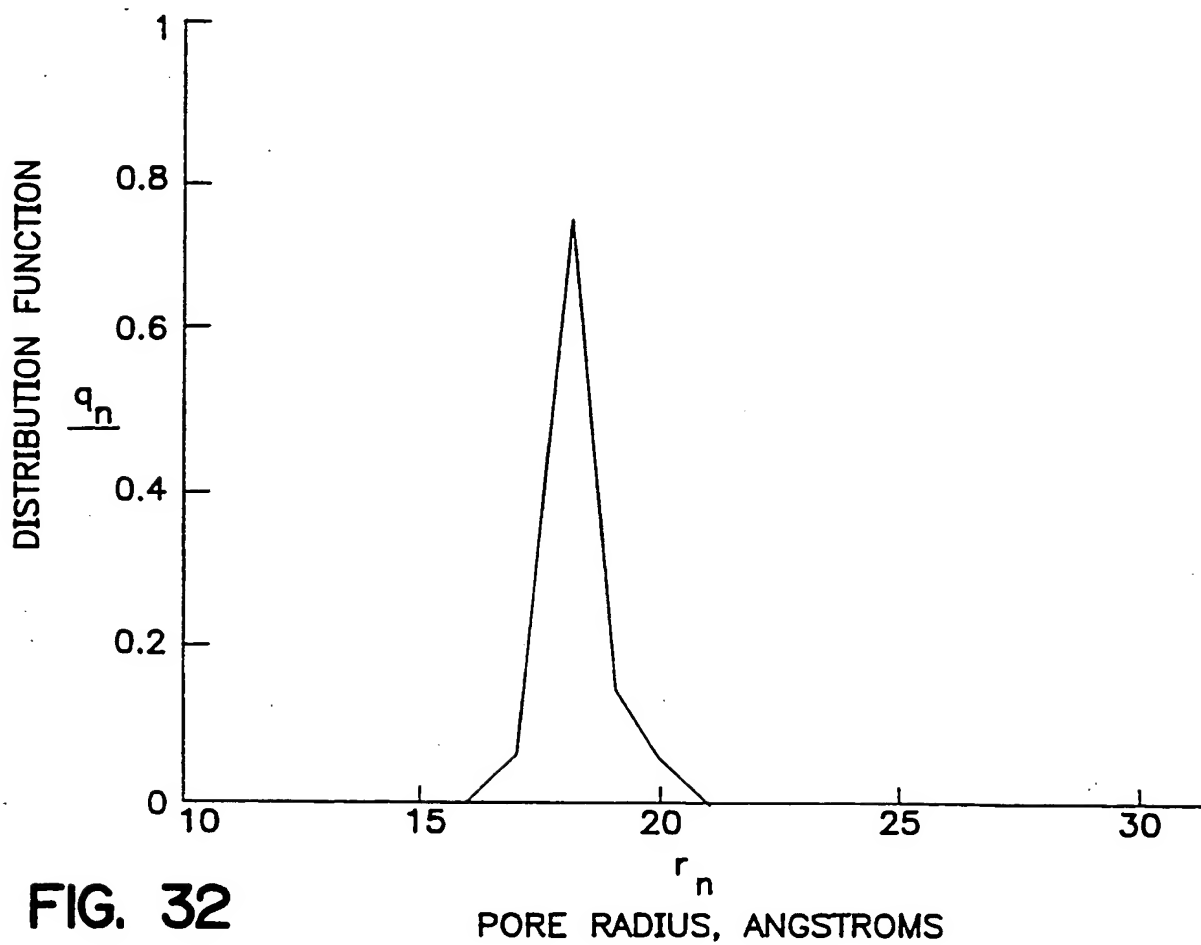


FIG. 32

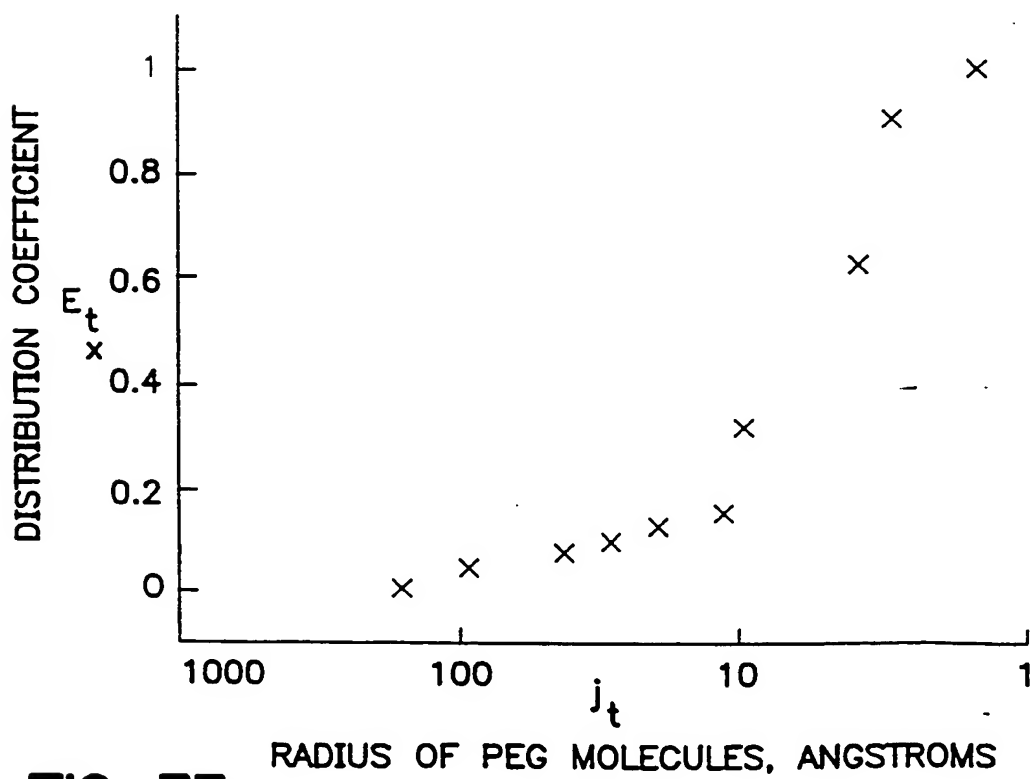


FIG. 33

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30/46

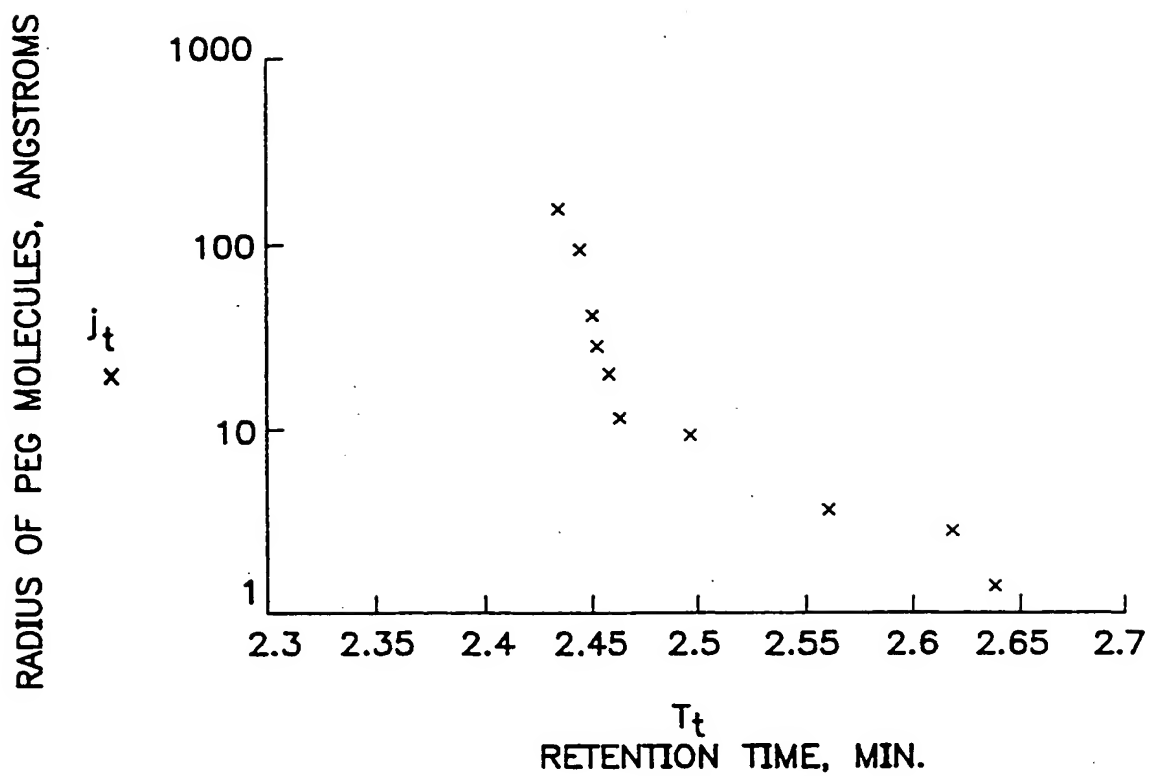


FIG. 34

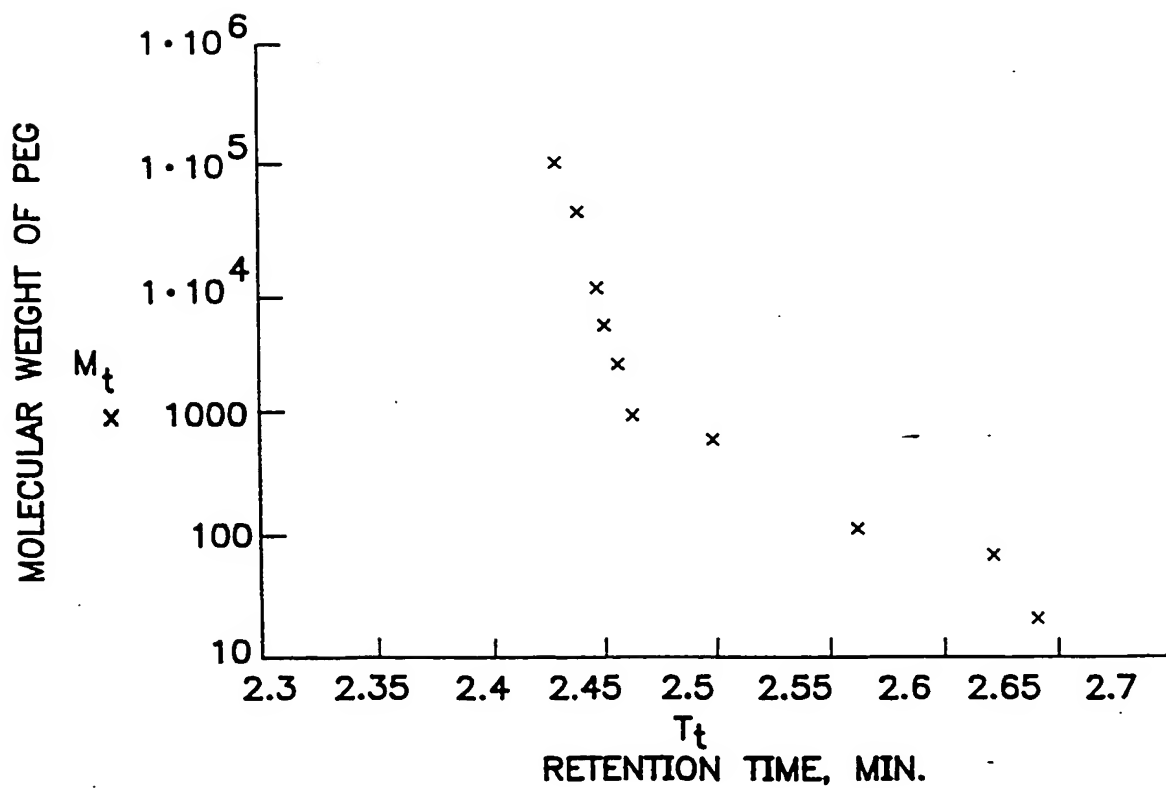


FIG. 35

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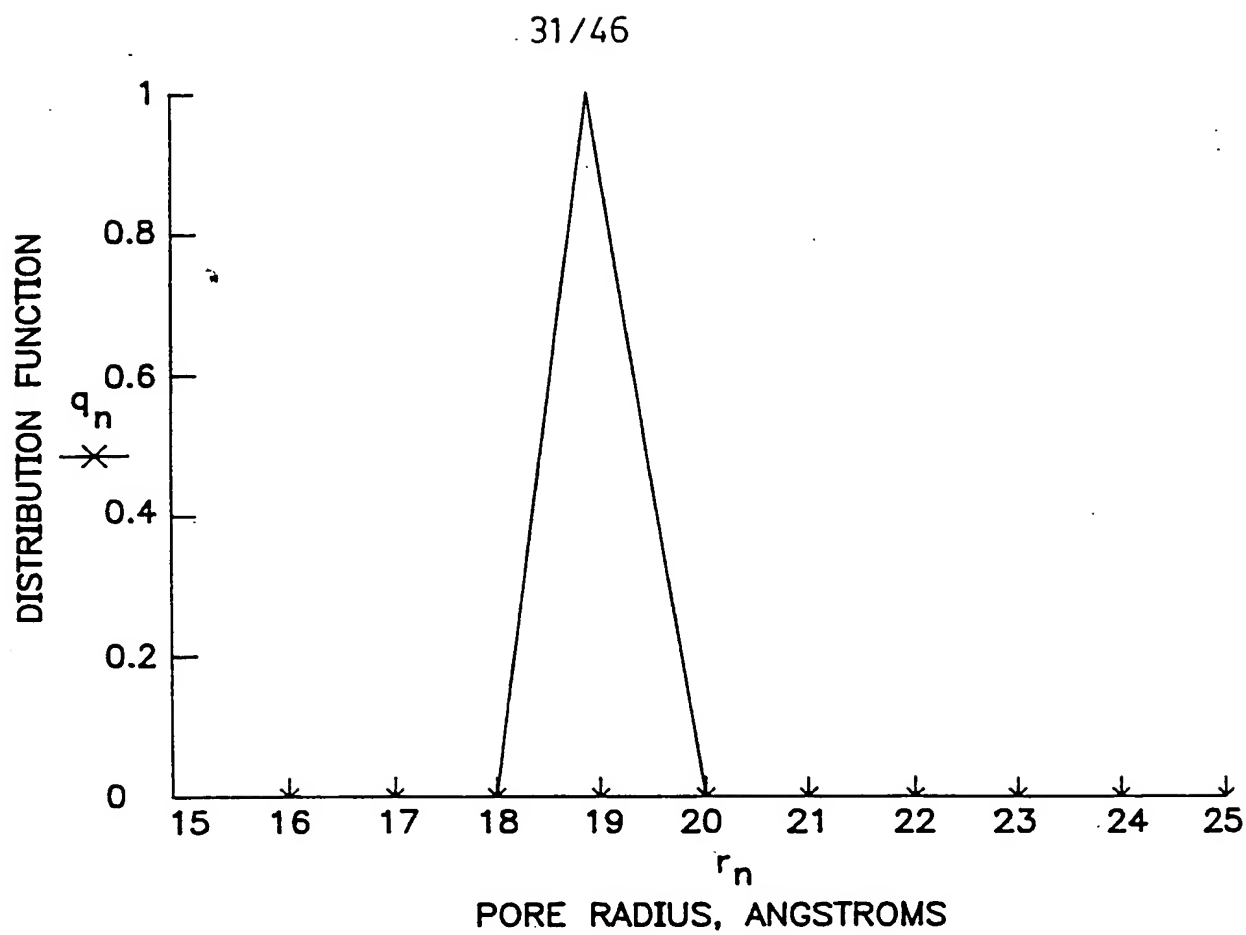


FIG. 36

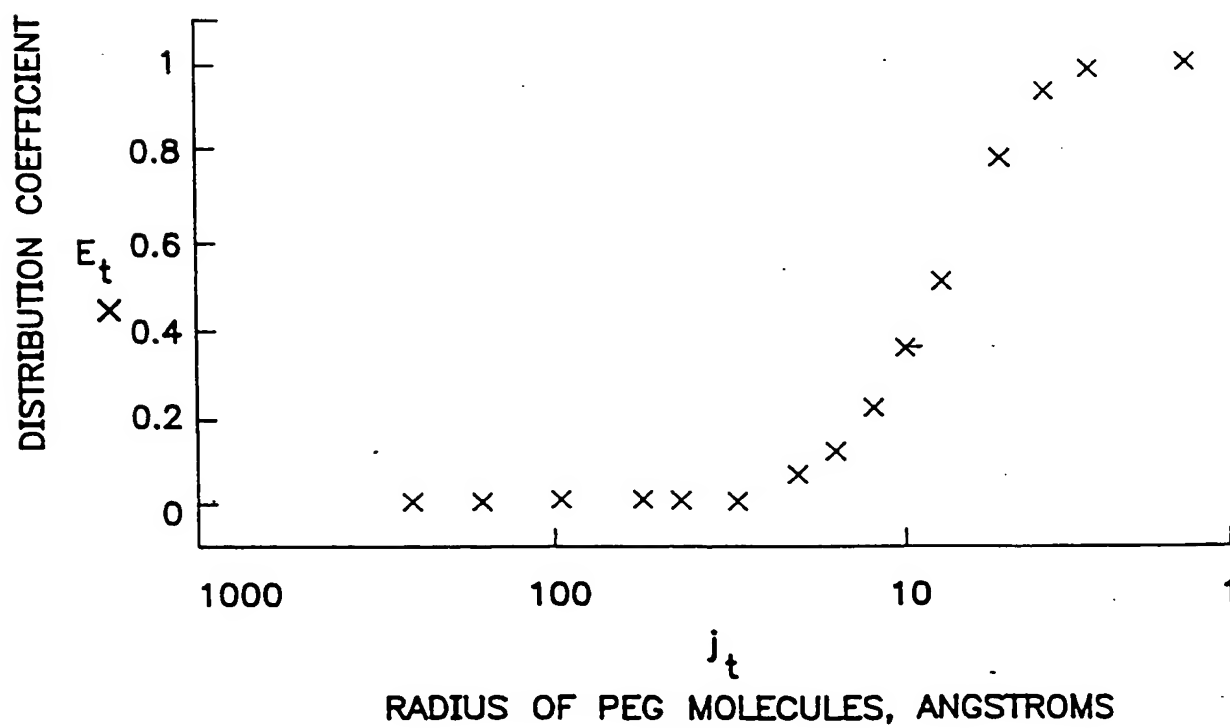


FIG. 37

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32/46

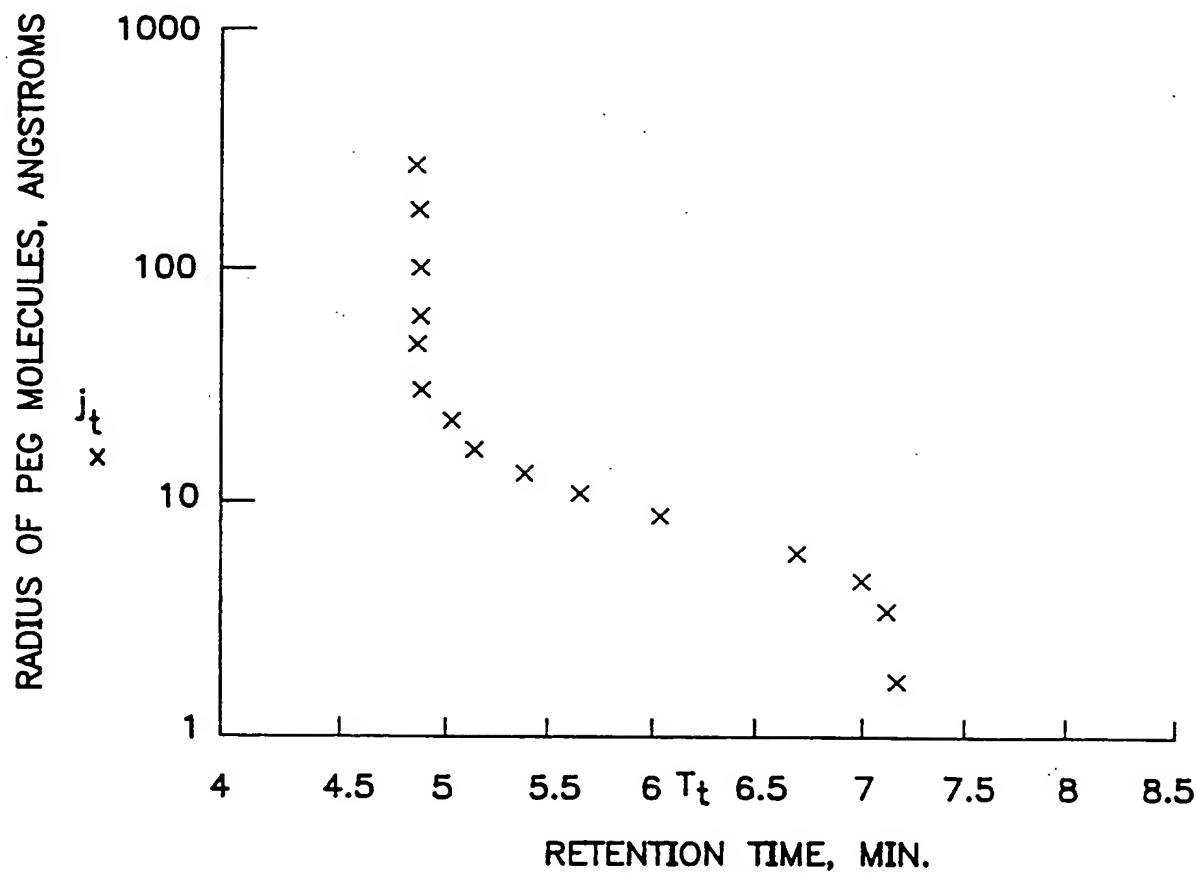


FIG. 38

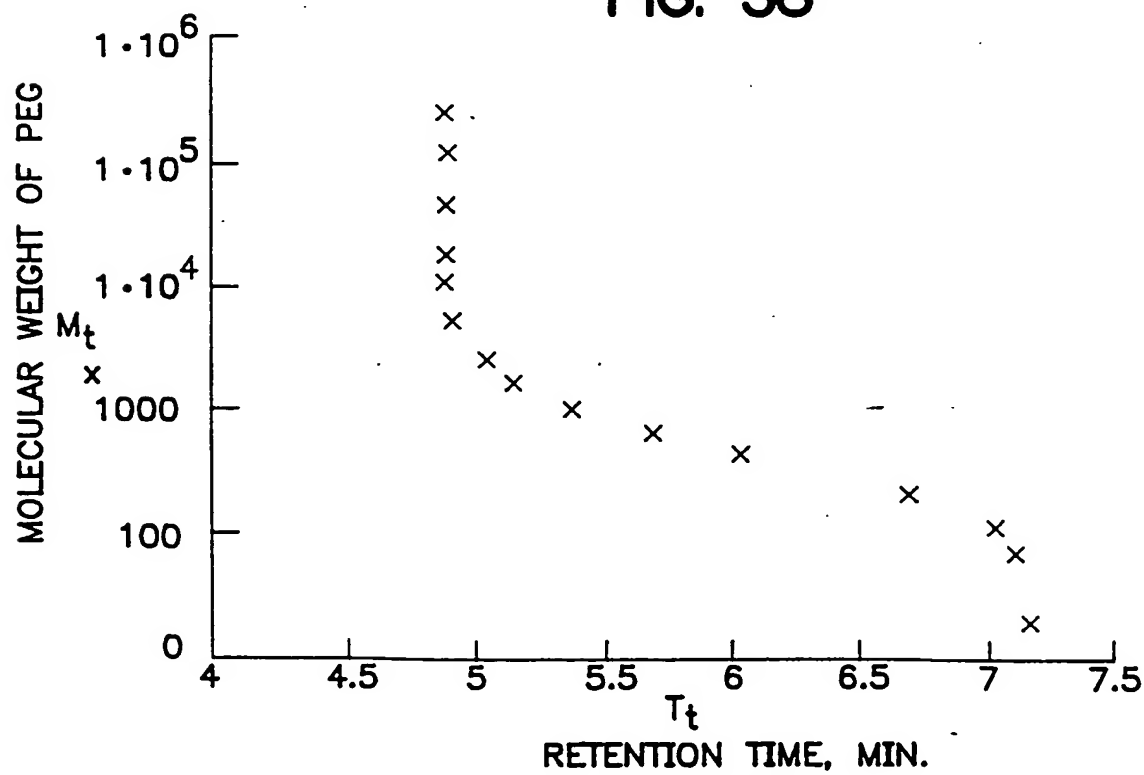


FIG. 39

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33/46

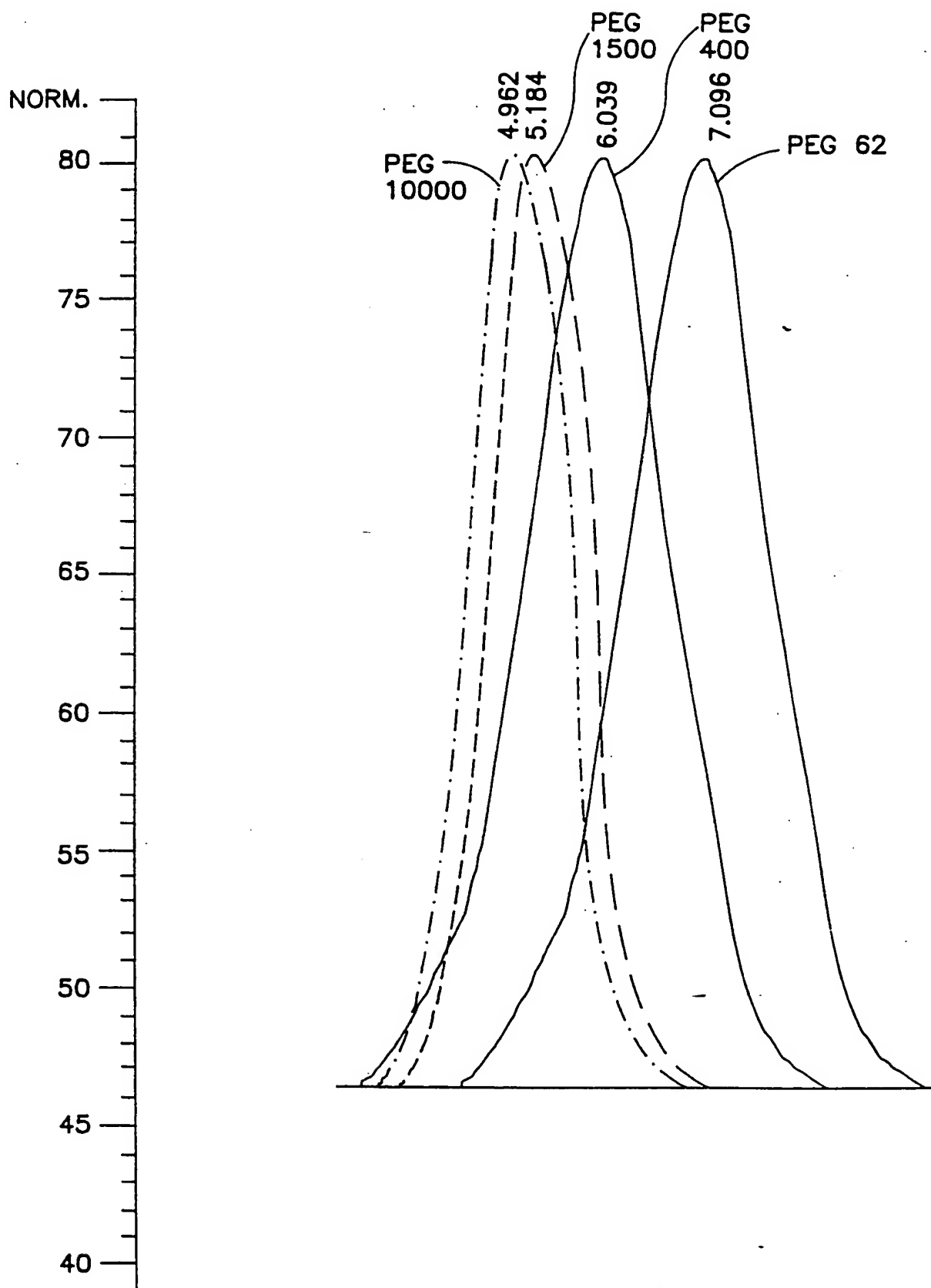


FIG. 40

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34/46

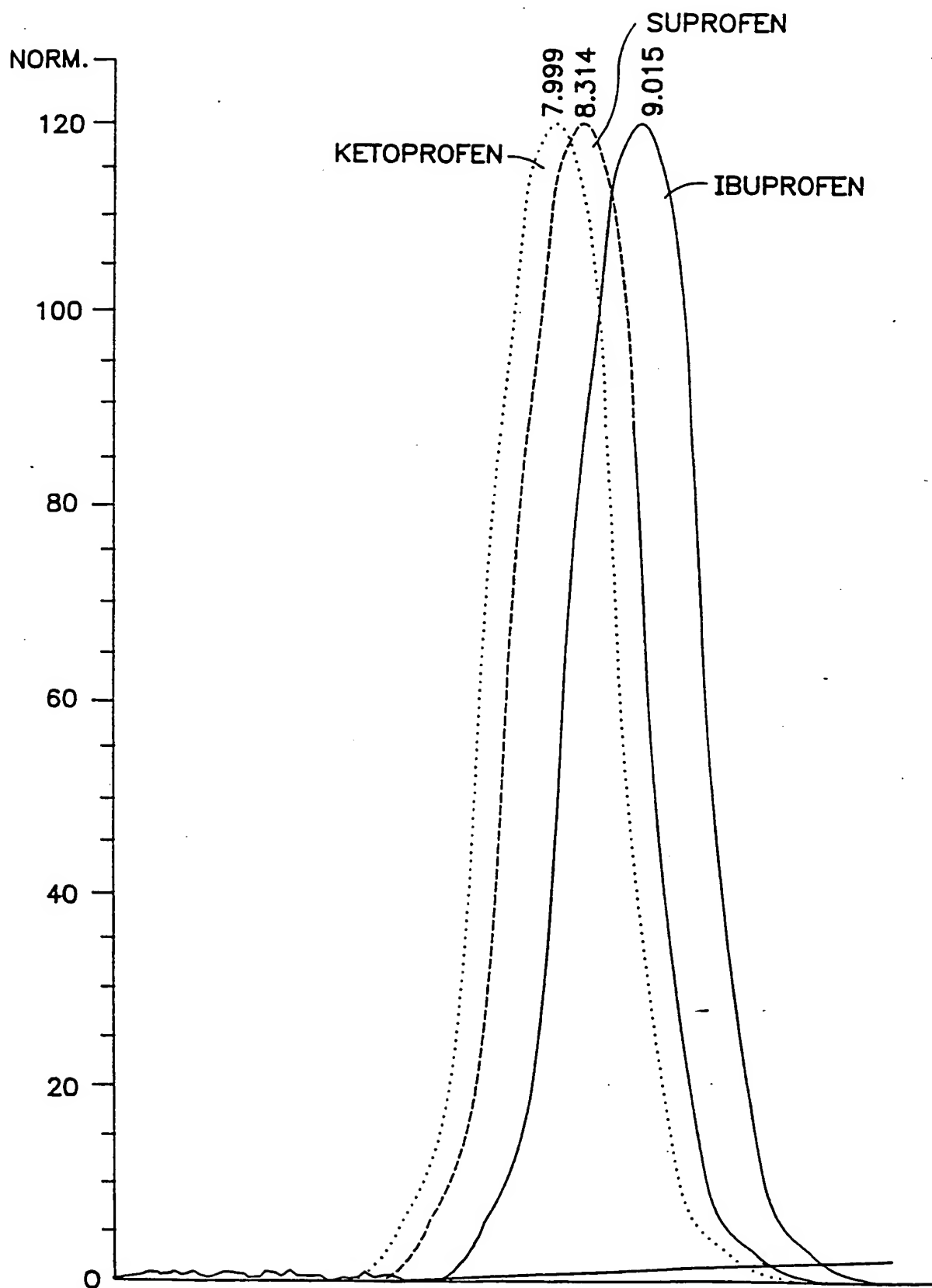


FIG. 41

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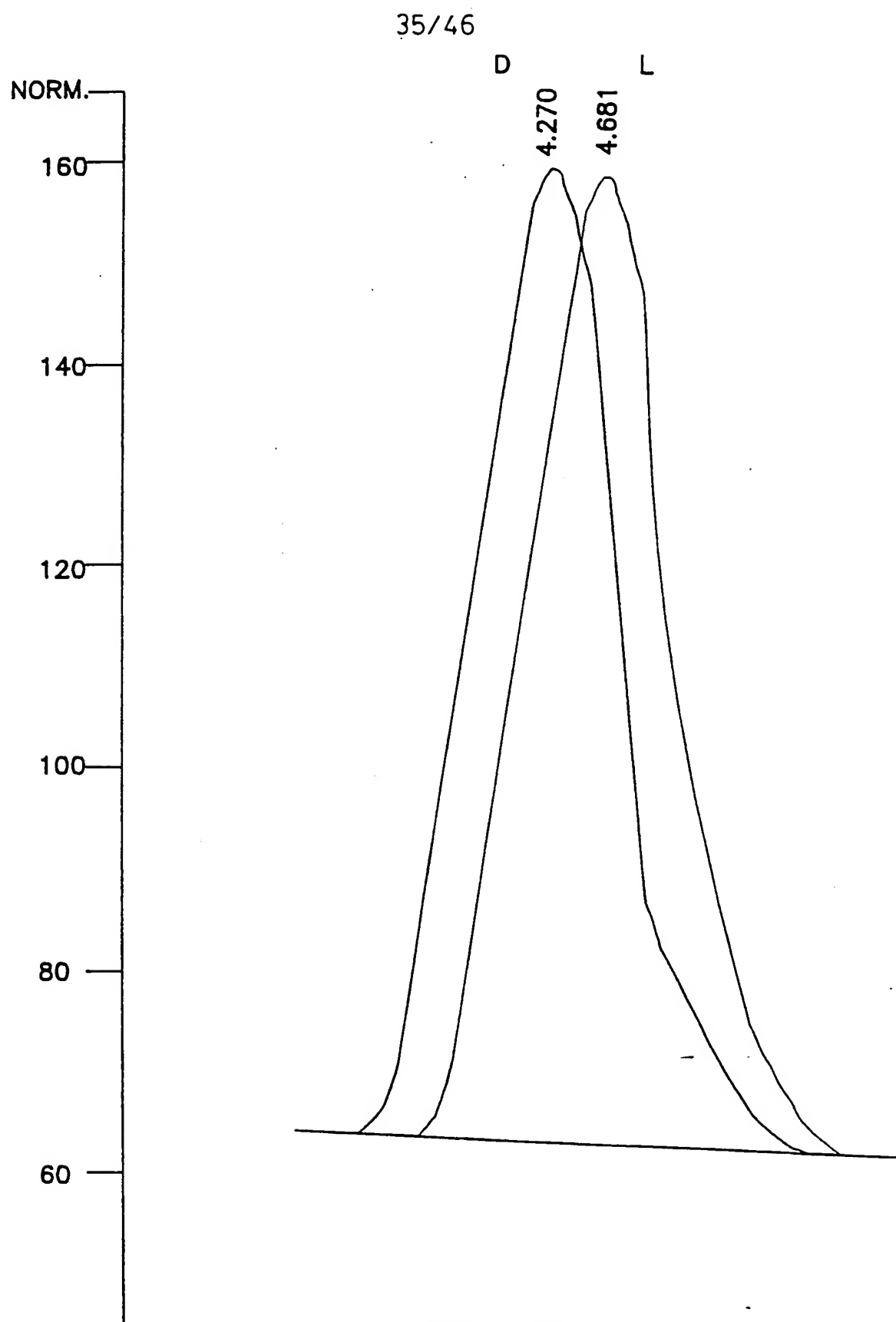
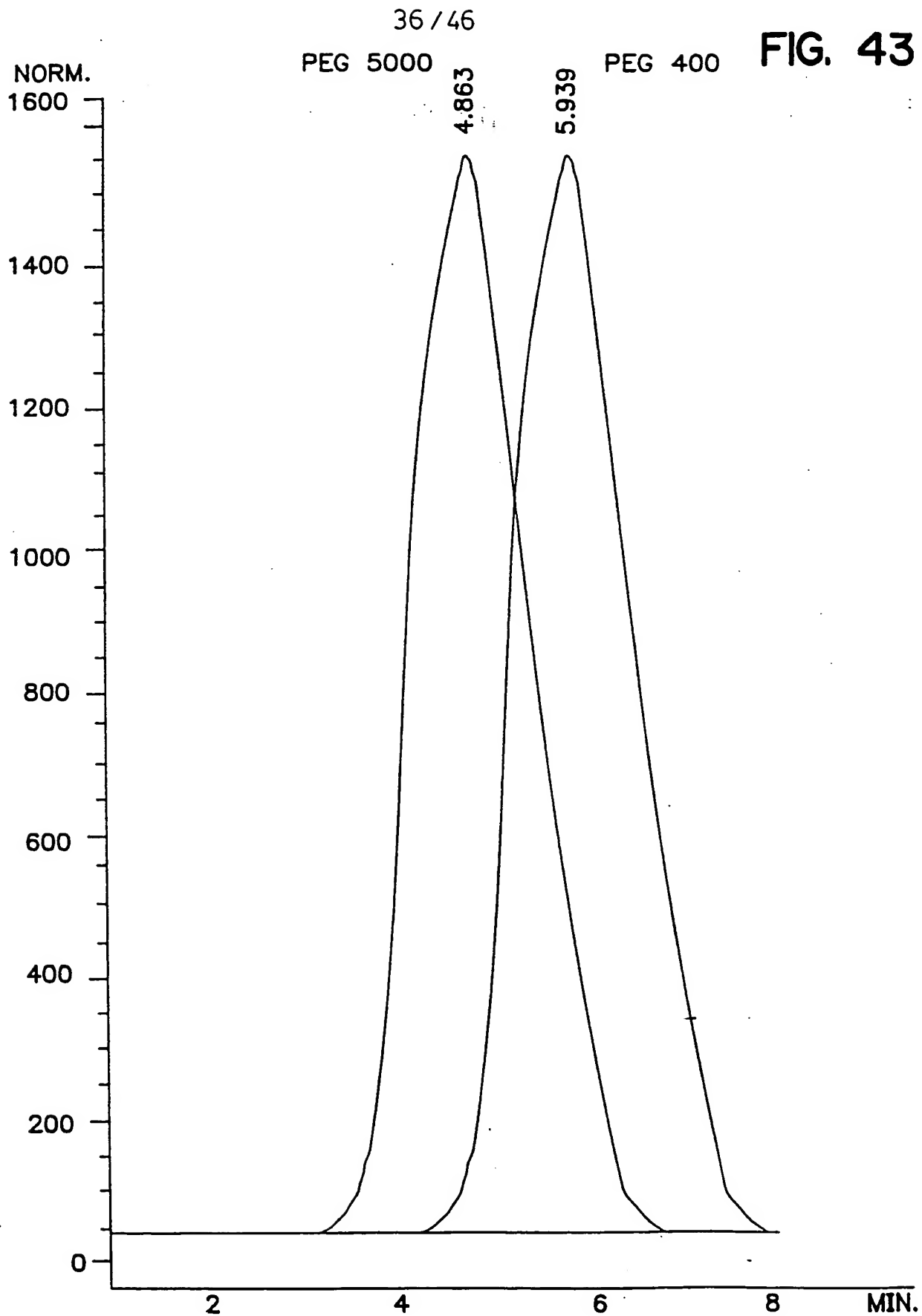


FIG. 42

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37/46

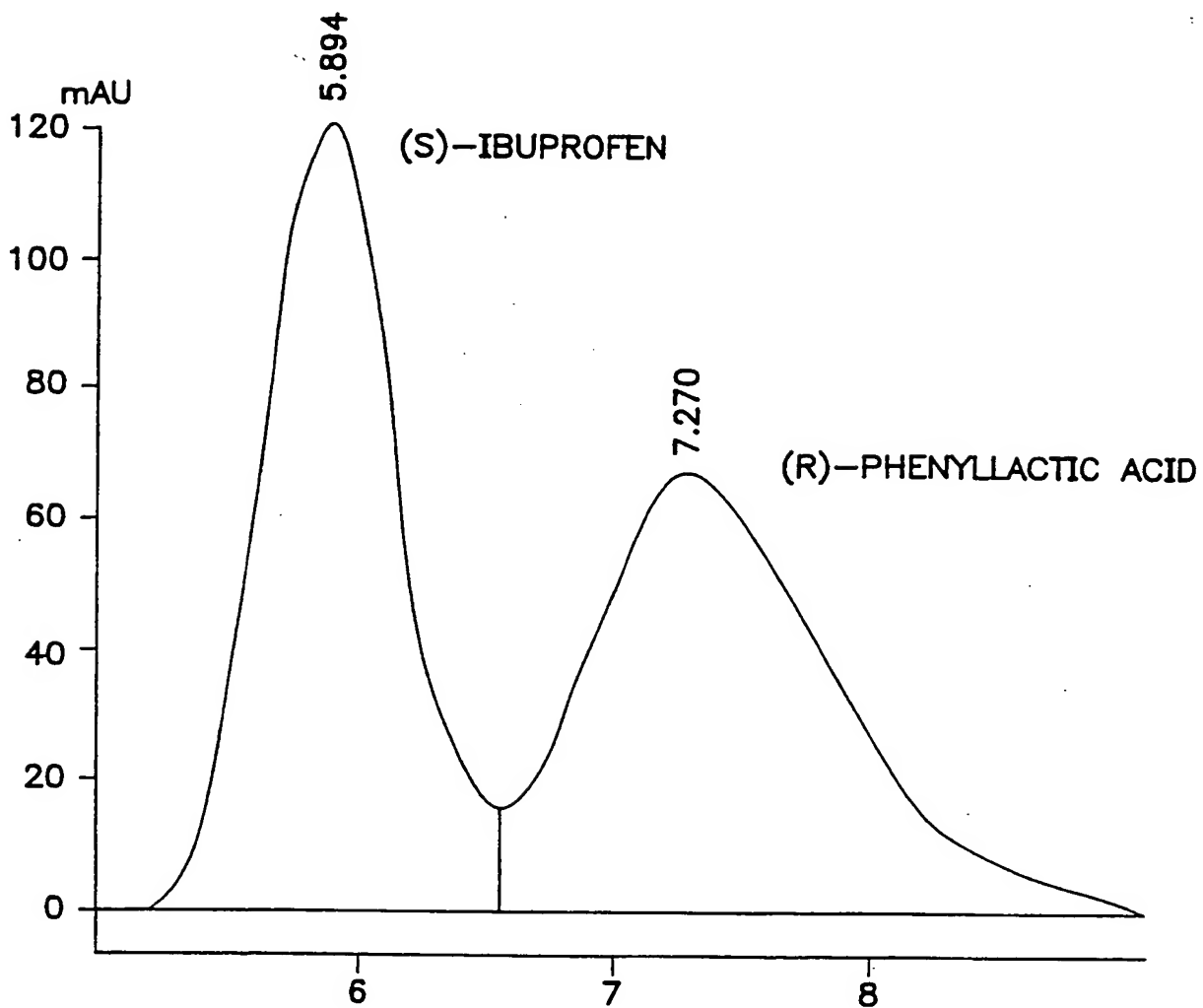


FIG. 44

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38/46

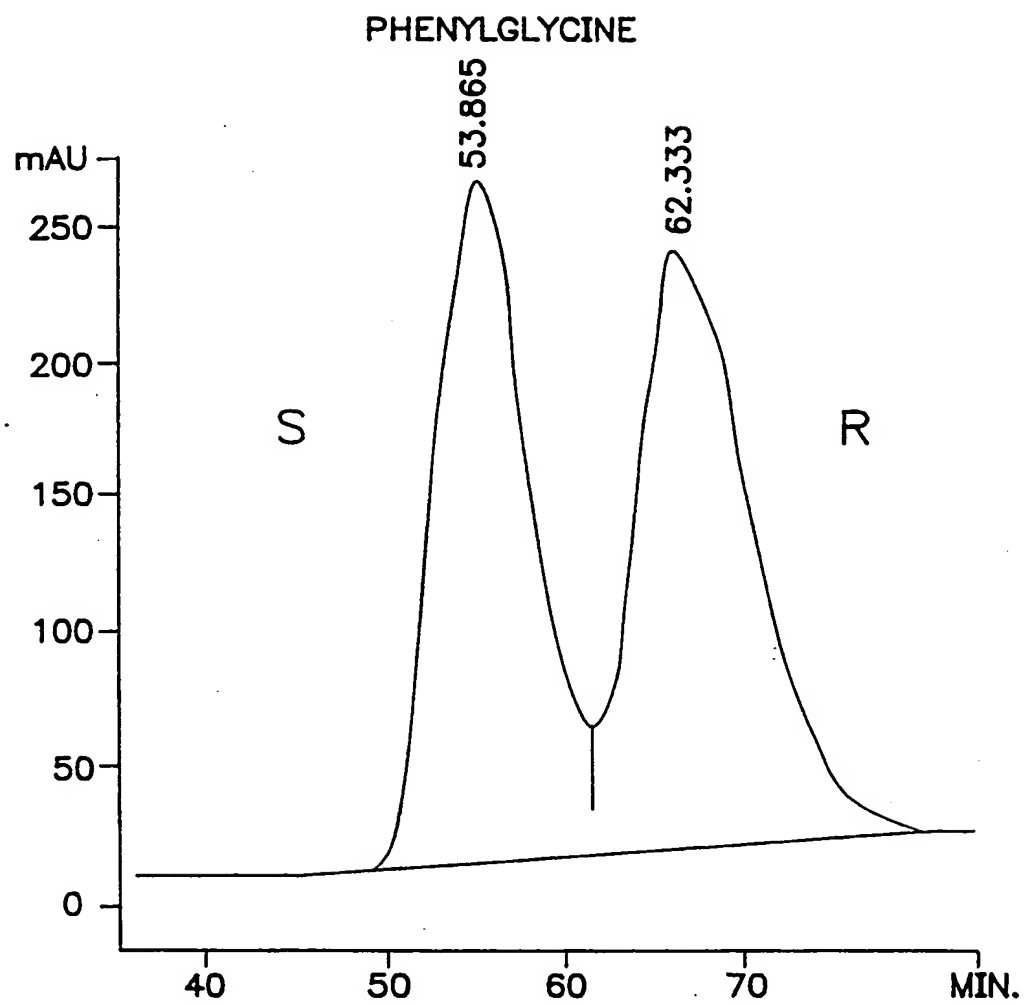


FIG. 45

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39/46

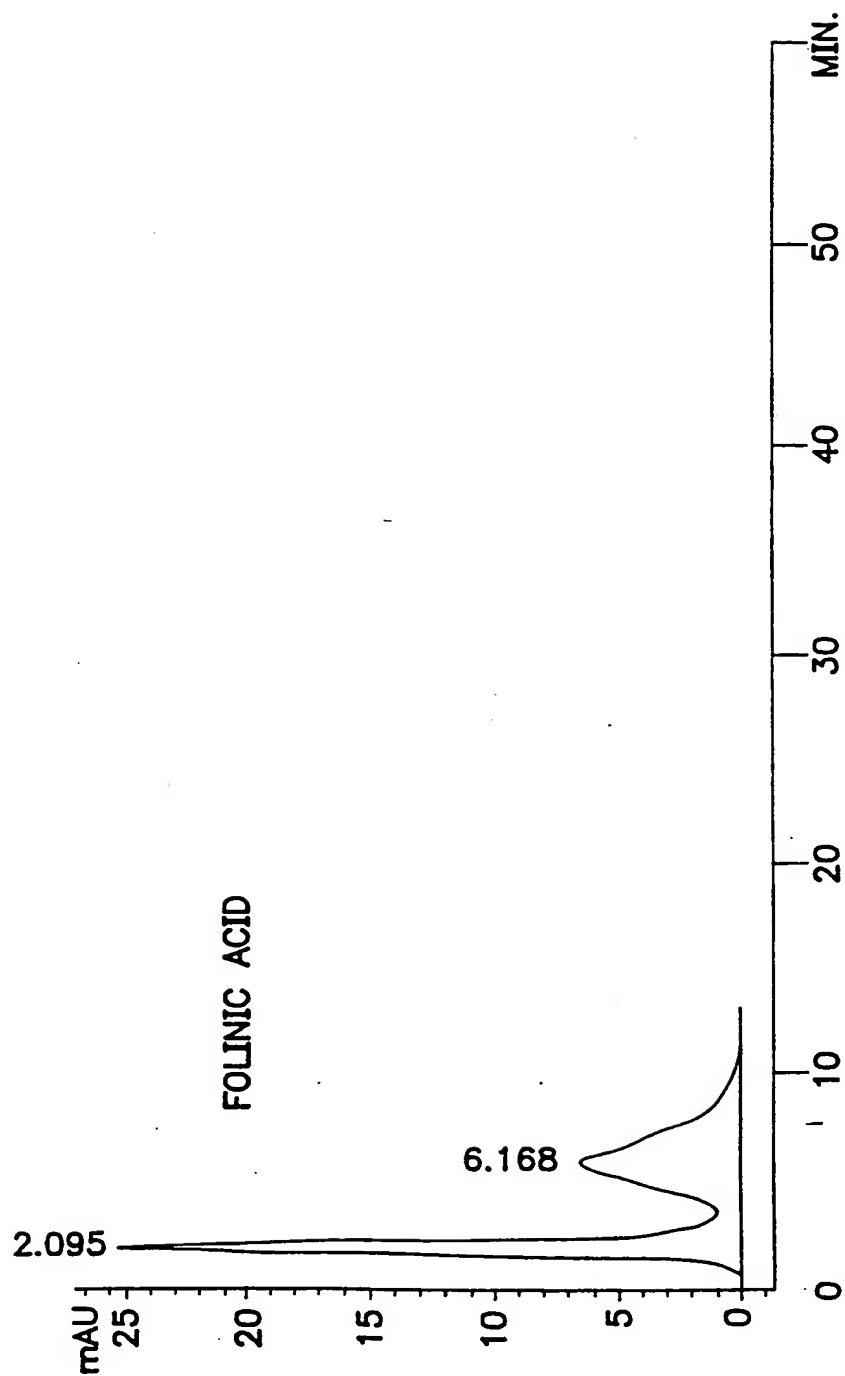


FIG. 46

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40/46

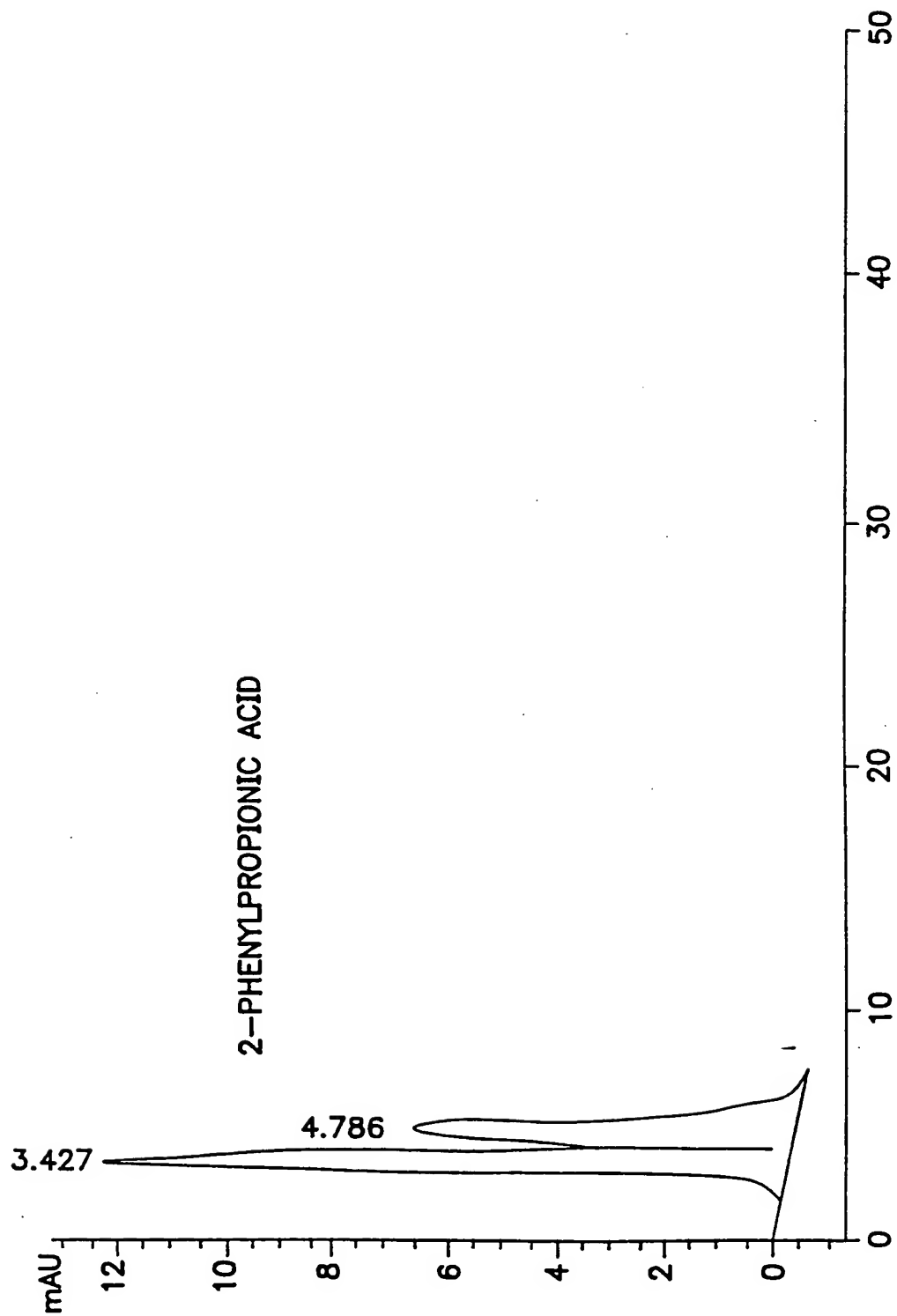


FIG. 47

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41/46

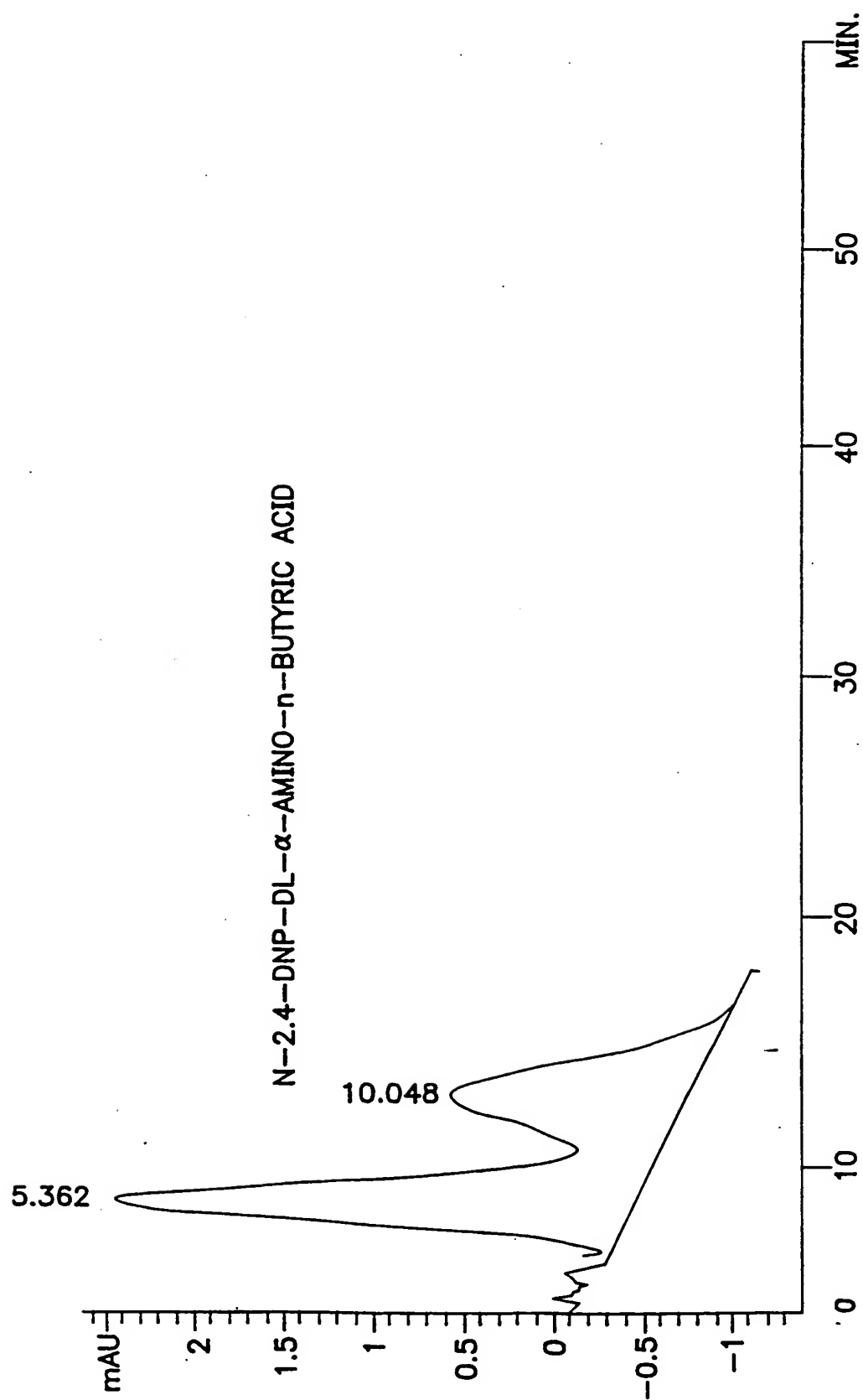


FIG. 48

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42 / 46

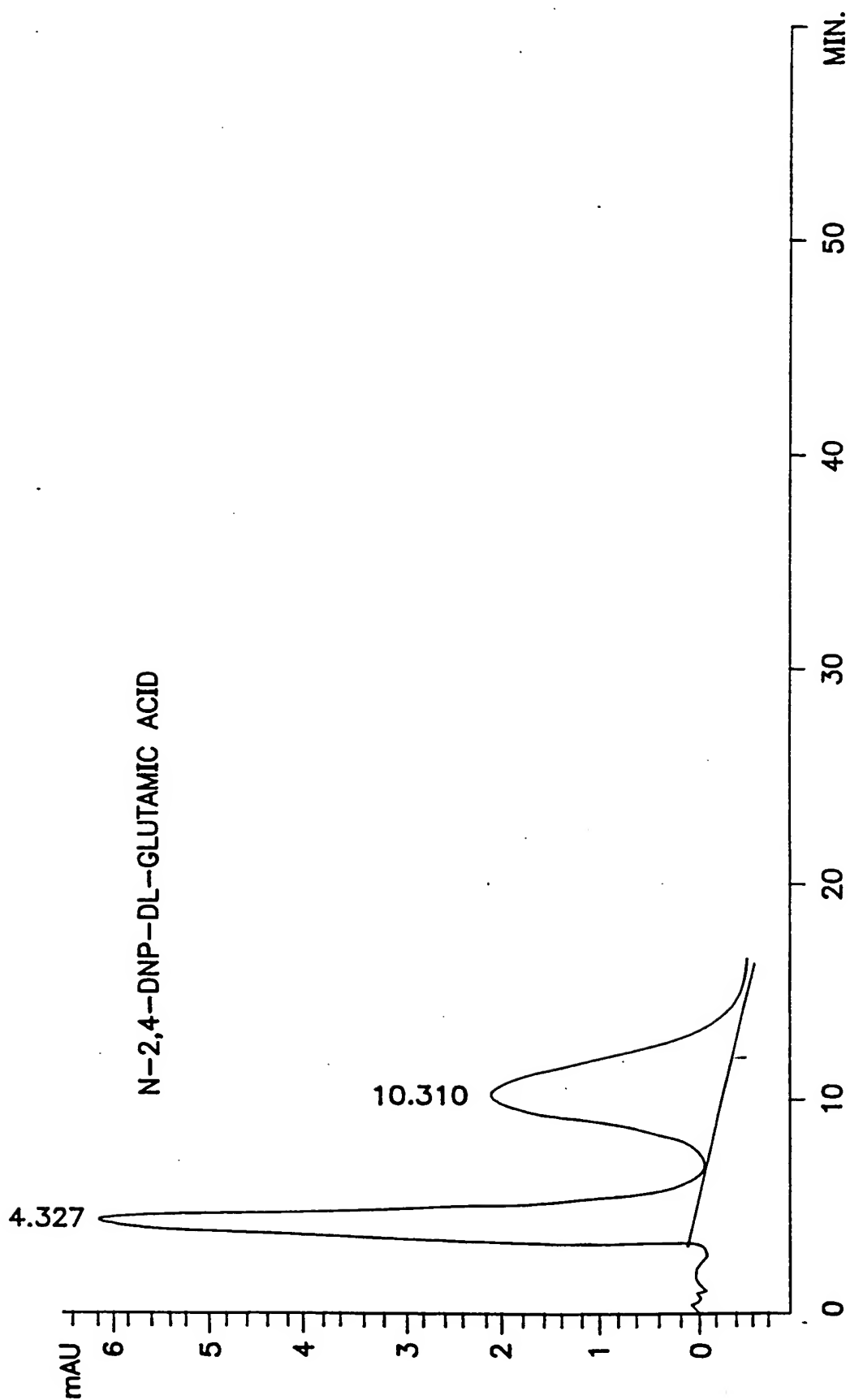


FIG. 49

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43/46

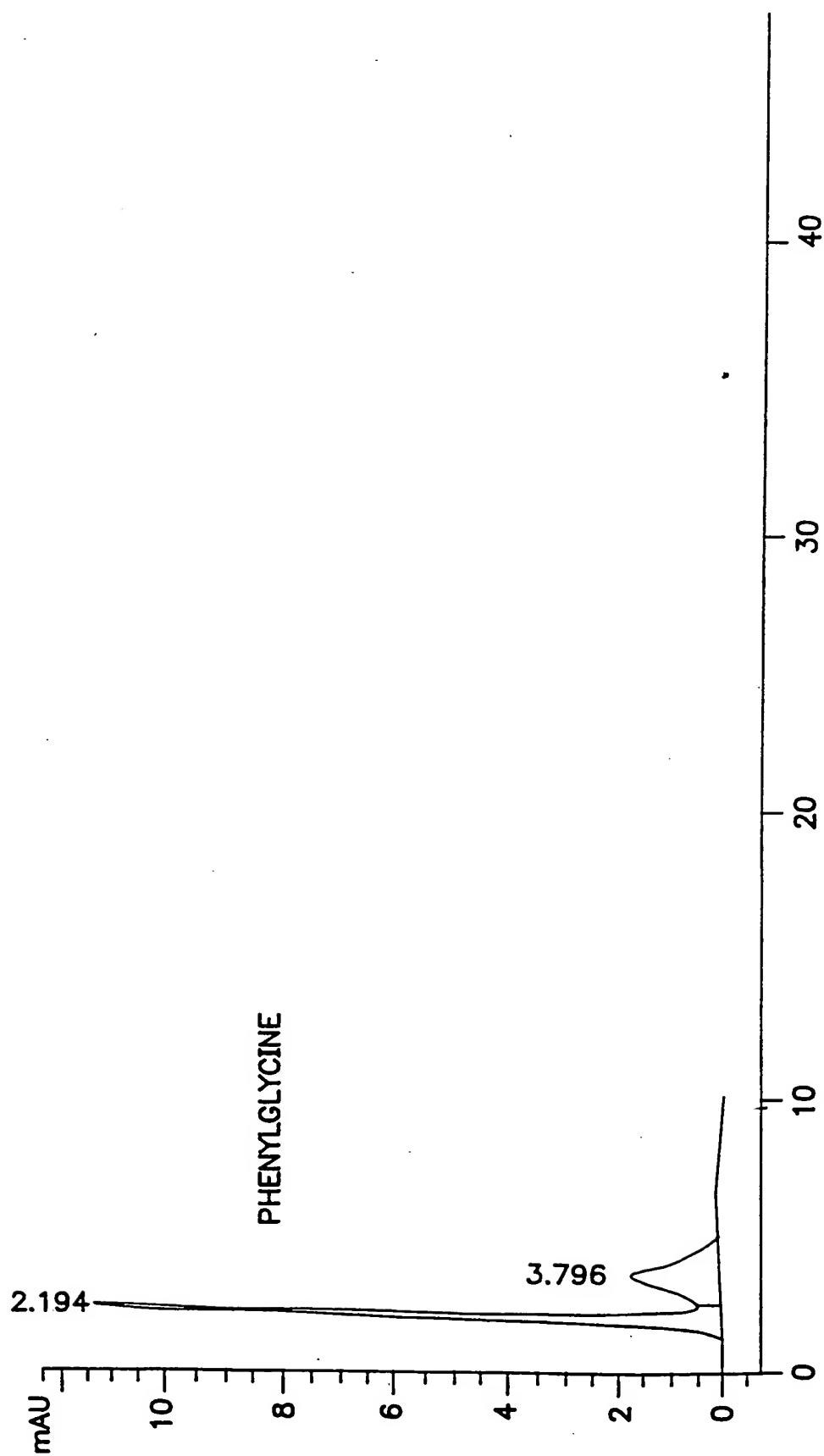


FIG. 50

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44/46

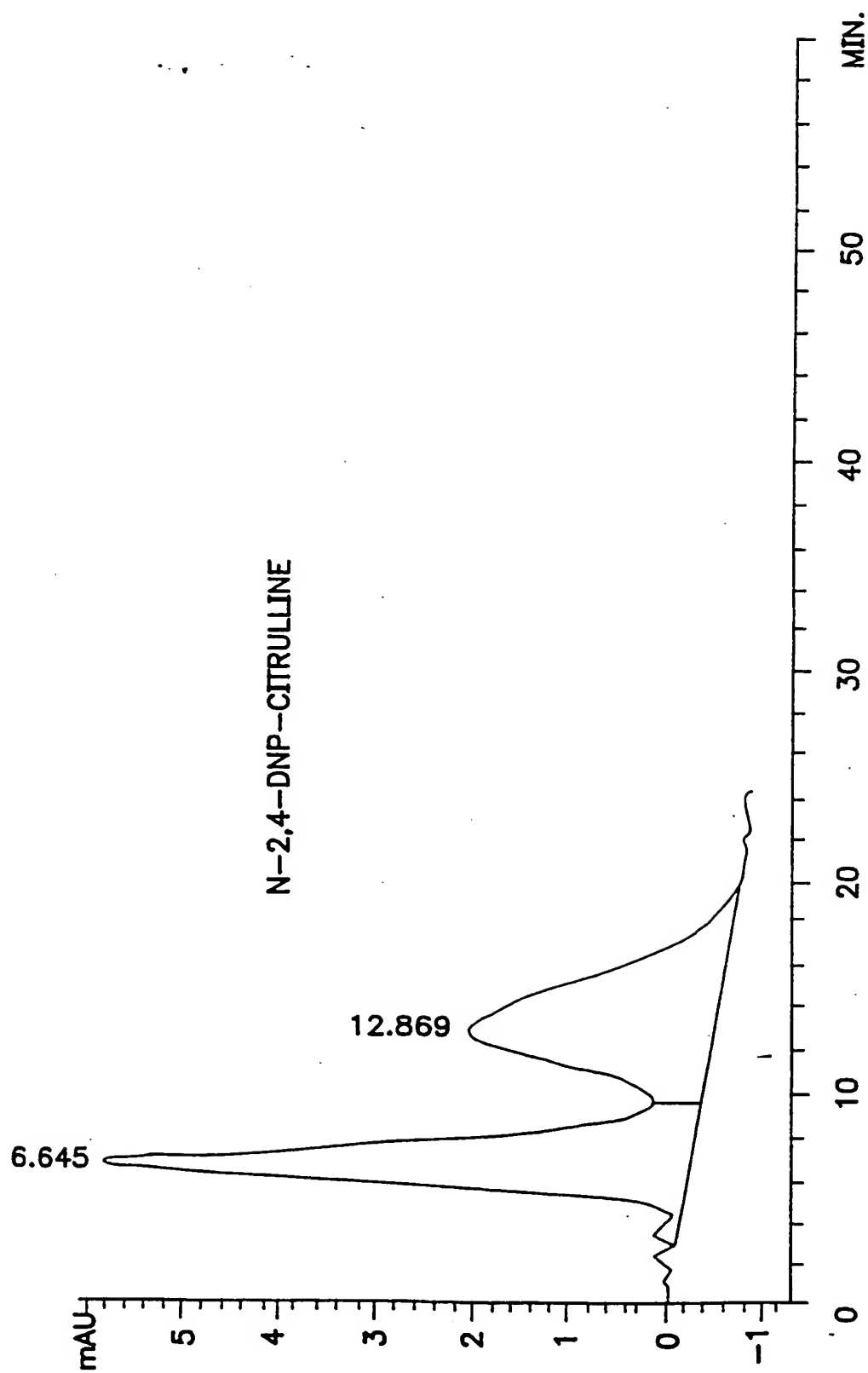


FIG. 5I

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45/46

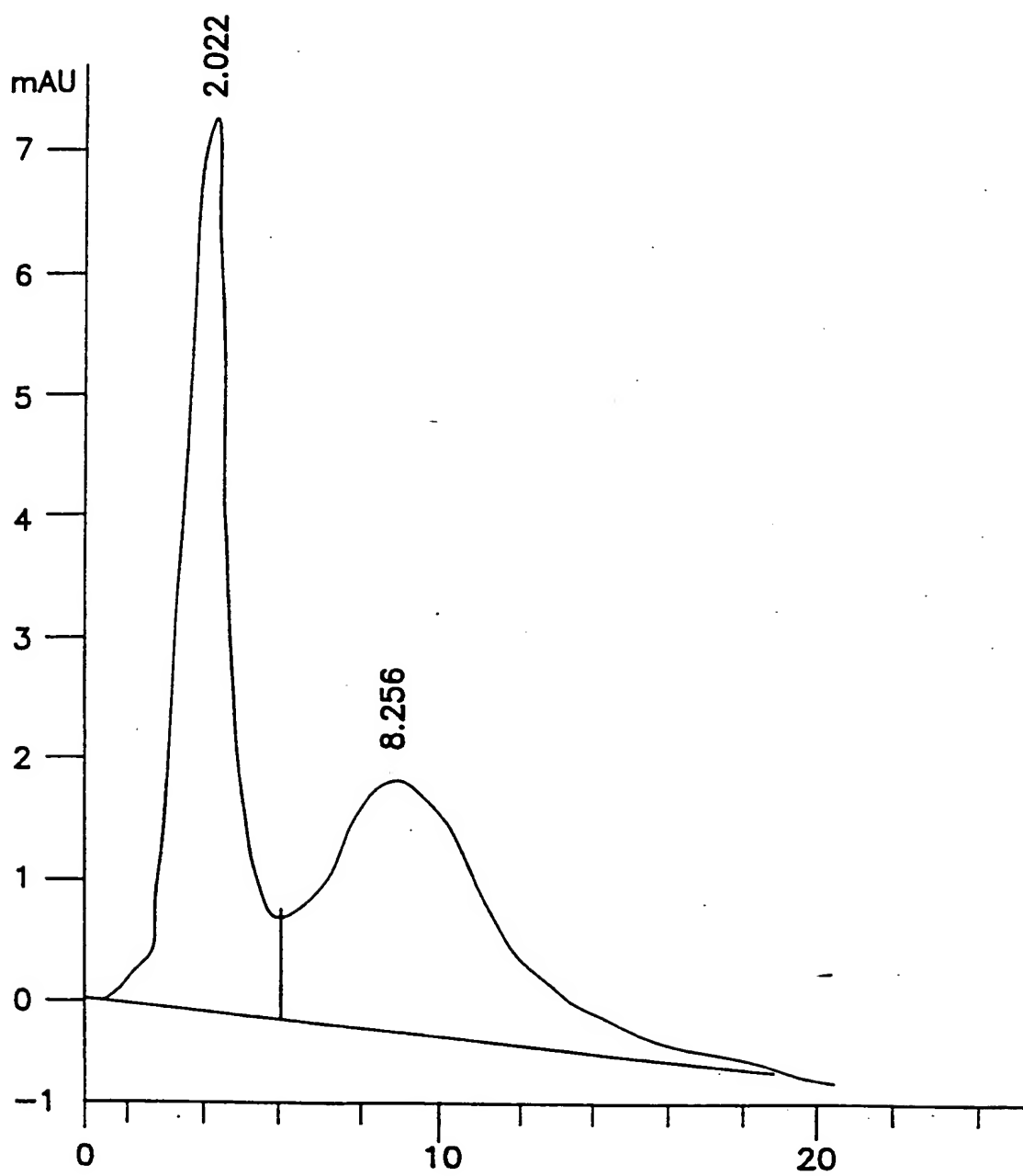


FIG. 52

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46 / 46

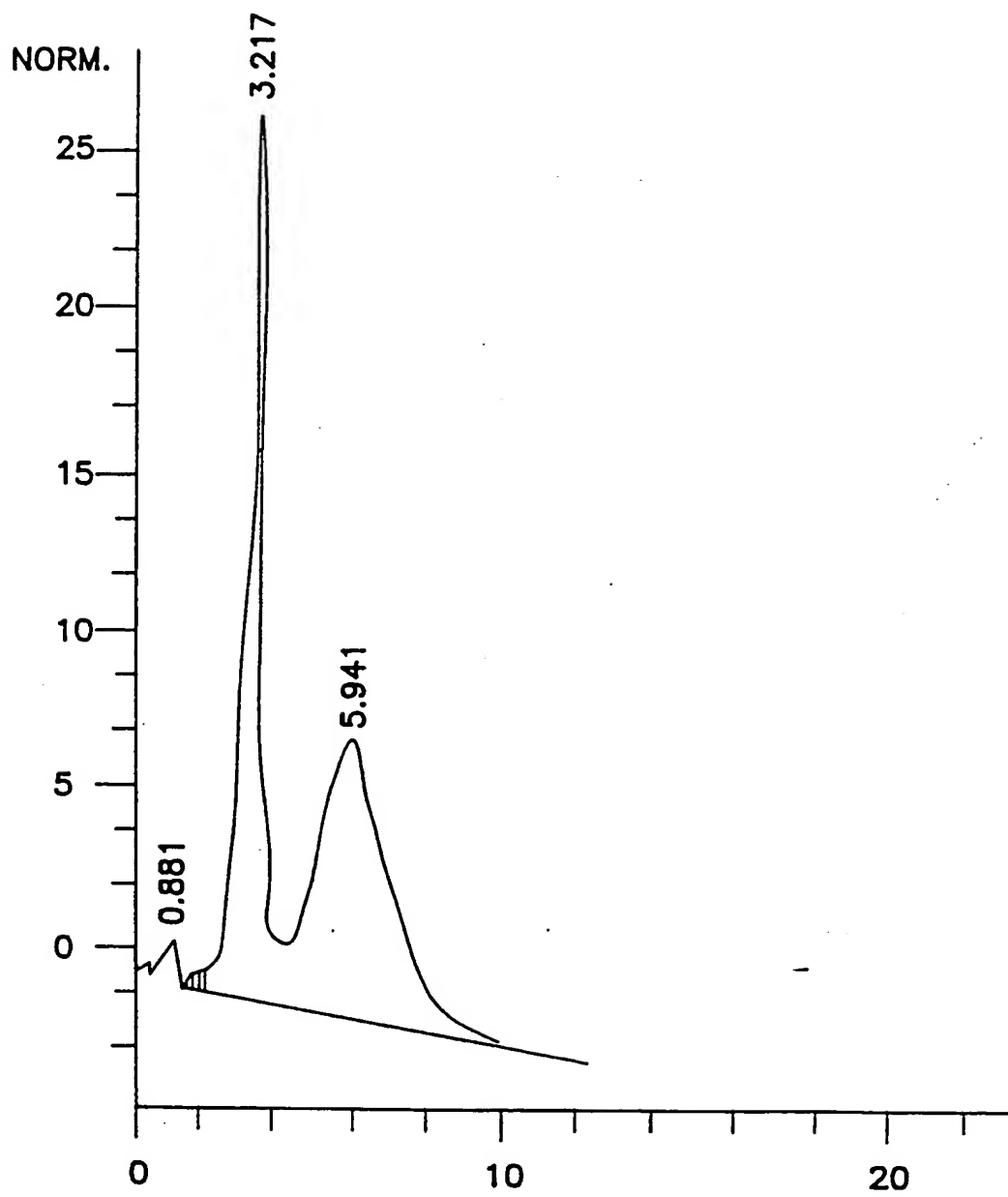


FIG. 53

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# INTERNATIONAL SEARCH REPORT

Inte. onal Application No

PCT/US 97/17167

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 6 B01D15/08 G01N30/48

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 B01D G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 95 09907 A (THE UAB RES. FOUND.) 13 April 1995 cited in the application  see page 9-10; claims 1-15 see page 8; example 5 ---	1-7, 10-12, 15-17, 23-28, 31, 40-45, 55,56
A	US 5 545 317 A (WILLIAMS) 13 August 1996 see column 7, line 35 - column 10, line 57 --- -/--	1-7, 9-14,18

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

27 January 1998

Date of mailing of the international search report

10/02/1998

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Authorized officer

Wendling, J-P

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International Application No.  
PCT/US 97/17167

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		Relevant to claim No.
Category	Citation of document, with indication, where appropriate, of the relevant passages	
A	WO 92 02617 A (VERTEX PHARMACEUTICALS) 20 February 1992  see page 40, line 14 - page 45, line 13 see page 87, line 89; claims 14-24 ---	1-3, 15-17, 19-22
A	US 5 512 169 A (WILLIAMS) 30 April 1996 ---	
P, A	US 5 559 039 A (WILLIAMS) 24 September 1996 ---	
P, A	CHEMICAL ABSTRACTS, vol. 125, no. 25, 16 December 1996 Columbus, Ohio, US; abstract no. 321216v, MARGOLIN: "CROSSLINKED ENZYME CRYSTALS AS NOVEL MATERIALS FOR CATALYSIS AND CHROMATOGRAPHY" page 617; column 1; XP002053385 & CHIMIA 1996, 50(9), 423-424 see abstract -----	1



# INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/US 97/17167

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9509907 A	13-04-95	AU 7926194 A	01-05-95
US 5545317 A	13-08-96	US 5512169 A	30-04-96
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		US 5545317 A	13-08-96
US 5559039 A	24-09-96	US 5512169 A	30-04-96
		US 5545317 A	13-08-96

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